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13. ABSTRACT ***(Maximum 200 Words)***

We have investigated the anti-cancer effects of retinoids, the natural and synthetic vitamin A derivatives, in breast cancer cells. Our data demonstrate that retinoids can effectively inhibit growth of breast cancer cells. The growth inhibitory effect of retinoids is mediated by their induction of retinoic acid receptor beta (RARβ) expression, expression, in addition to their suppression of mitogenic effect of estrogen receptor and inhibition of AP-1 activity. Expression of RARβ inhibits growth of breast cancer cells by promoting their apoptosis. Our study also show that retinoid activity in brease cancer cells is regulated by BAG-1, a recently identified cell survival gene that interacts with Bcl-2, providing a possible molecular basis for interaction between retinoid and apoptosis signaling. In studying regulation of RARβ expression, we demonstrate that two retinoid signaling pathways, i.e. the RXR-dependent pathway and the RAR-dependent pathway, can induce RARβ expression and growth inhibition. In addition, we have found that lack of COUP-TF expression is mainly responsible for loss of RARβ expression in retinoid-resistant breast cancer cells. These results largely enhance our understanding of the mechanism of retinoid action in breast cancer cells and also point to a possibility of restoring or enhancing retinoid activity in breast cancer cells.

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INTRODUCTION

Retinoids, a class of natural and synthetic vitamin A analogs, exert profound effects on many biological processes, including proliferation and differentiation, vision, reproduction, morphogenesis, and pattern formation. They are also promising chemopreventive agents against various types of cancers, including breast cancer. In animals, administration of retinoids inhibits the initiation and promotion of mammary tumors induced by carcinogens. Their efficacy against mammary carcinogenesis in animal models has been demonstrated by their ability to increase the latency period for tumor appearance and decrease the number of animals with cancer (1). In vitro, retinoids were shown to inhibit the growth of human breast cancer cells, majority of which, however, are hormone-dependent estrogen receptor (ER) positive breast cancer cells. Inhibition on growth of such cells in culture has been observed when retinoids are administered alone or in combination with anti-estrogen, where synergistic effects are observed. Despite their cancer preventive effects, retinoids have not yet caused major clinical responses in clinical trials (2, 3), implying that their anti-cancer effectiveness diminishes in more malignant breast cancer cells and leads to one of the major drawbacks in retinoid therapy, the retinoid resistance. The ineffectiveness of retinoids in the treatment of patients with advanced breast cancer is consistent with in vitro observations that the anti-cancer effects of retinoic acid (RA) are mainly seen in estrogen-dependent breast cancer cells and that, upon progression of the disease to estrogen independence, breast cancer cells become refractory to RA. This also suggests the possible modification of retinoid response during progression of breast tumor. Further evaluation of retinoids for breast cancer treatment, therefore, is largely dependent on a better understanding of the molecular mechanism by which they act as anti-breast cancer agents.

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors (RARs) and retinoid X receptors (RXRs). 9-*cis* RA is a high affinity ligand for both RARs and RXRs, whereas all-*trans*-RA is a ligand for only RARs. RARs and RXRs are encoded by three distinct genes (α, β and γ) and are members of the steroid/thyroid hormone receptor superfamily, that function as ligand-activated transcription factors. RARs and RXRs primarily function as RXR/RAR heterodimers that are activated by RAR ligands. Binding of RXR ligands can induce RXR homodimer formation and function (6) and may be required for activation of certain RXR-containing heterodimers, such as RXR/LXR (7) and RXR/nur77 (8, 9). The dimeric complexes of retinoid receptors function as transcriptional factors that bind to a variety of RA-response elements (RAREs) on target genes and regulate the transcriptional expression of the genes. One of the most potent target genes identified so far is RARβ gene. RARβ is activated by RA through a RA response element (βRARE) in its promoter (10). The auto-induction of RARβ expression may play a critical role in amplifying retinoid responses. Alteration of retinoid receptor activity could be associated with neoplastic transformation as demonstrated by abnormal RARα transcripts found in patients with acute promyelocytic leukemia (APL).

Regulation of gene expression either positively or negatively by nuclear hormone receptors is modulated by additional cofactors that appear to provide a direct link to the core transcriptional machinery and to modulate chromatin structure (11). Some of these cofactors (coactivators) enhance transactivation by several nuclear receptors in the presence of their cognate ligands, whereas others (corepressors) are necessary for unliganded receptors to silence the activity of target promoters, and are dissociated upon binding of ligand to the receptors. In addition to receptor coactivators and corepressors, a number of other cellular proteins, such as AP-1, have been implicated in the regulation of nuclear hormone receptor activity, probably through their interaction with receptors (12).

Despite intensive research, how the inhibitory effect of retinoids on breast cancer cell growth was mediated and how this inhibitory effect was lost in hormone-independent estrogen receptor-negative breast cancer cells were largely unclear. The major goal of the proposed experiments was to understand the molecular mechanism by which retinoids exert their anti-cancer effects in breast cancer cells. The specific aims of this project are: 1) to analyze the anti-estrogen effect of retinoid receptors; 2) to characterize proteins that interact with RXR; 3) to analyze transactivation and anti-AP-1 activities of retinoid receptors; 4) to analyze the mechanism by which retinoid receptor activities are impaired, and 5) to analyze the function of RXR homodimers.

BODY

Interaction between retinoid and estrogen signalings.

The observation that the growth inhibitory effect of RA was mainly seen in estrogen-dependent ER-positive breast cancer cells but not in estrogen-independent ER-negative cells suggested that ER functions as a target of retinoid action. We propose that the anti-estrogen effect of retinoids may be one of the major mechanisms by which they act to inhibit breast cancer cell growth and that the loss of retinoid sensitivity during progression of breast tumor may be due to alterations of retinoid receptor activities. To study the possible interaction between ER and RAR signalings, we investigated molecular mechanism by which RAR and ER regulated expression of lactoferrin, which was known to be regulated by both RA and estrogen (**Lee et al., Mol. Cell. Biol. 15, 4194-4207, 1995**). By mutational analysis, we identified a RARE in the 5'-flanking region of the lactoferrin gene promoter. The lactoferrin-RARE is composed of two AGGTCA-like motifs arranged as a direct repeat with one base pair spacing (DR-1). Gel retardation assay demonstrated that it bound strongly with RXR homodimers and RXR/ RAR heterodimers. In CV-1 cells, the lactoferrin-RARE linked with a heterologous thymidine kinase (tk) promoter was strongly activated by RXR homodimers in response to 9-cis RA but not to all-trans RA. An unique feature of the lactoferrin-RARE is that it shares an AGGTCA-like motif with an estrogen responsive element (ERE) (Figure 1). The composite RARE/ERE contributes to the functional interaction between retinoid receptors and ER and their ligands. In CV-1 cells, cotransfection of the retinoid and estrogen receptors led to a mutual inhibition of each other's activity while a RA-dependent inhibition of ER activity was observed in breast cancer cells (Figure 1). Thus, the lactoferrin RARE/ERE functions as a signaling switch modular that mediates multihormonal responsiveness in the regulation of lactoferrin gene expression. Importantly, this study demonstrates a novel mechanism by which retinoid and estrogen signalings cross-talk. We have further extended this observation and shown that inhibition of estrogen-induced ER transactivation function can be observed even on a putative ERE in various breast cancer cells. These results, therefore, suggest that inhibition of ER activity may represent an important mechanism by which retinoids inhibit the growth of hormone-dependent ER-positive breast cancer cells.

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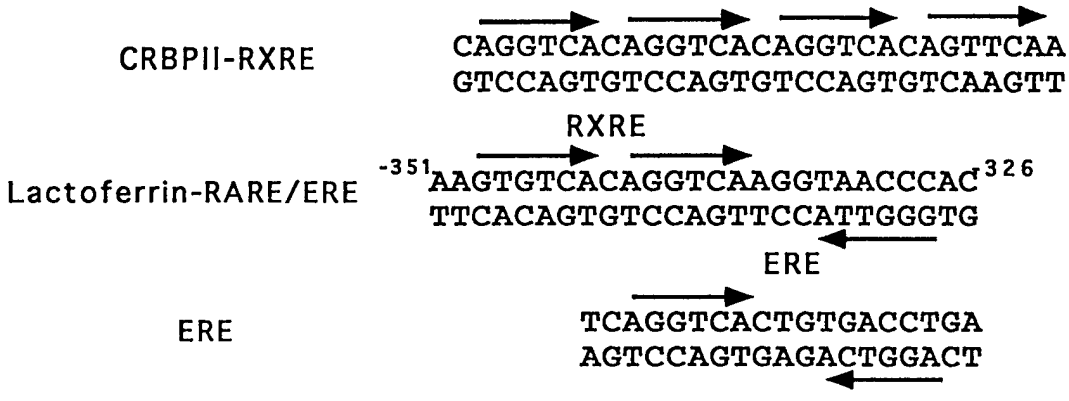

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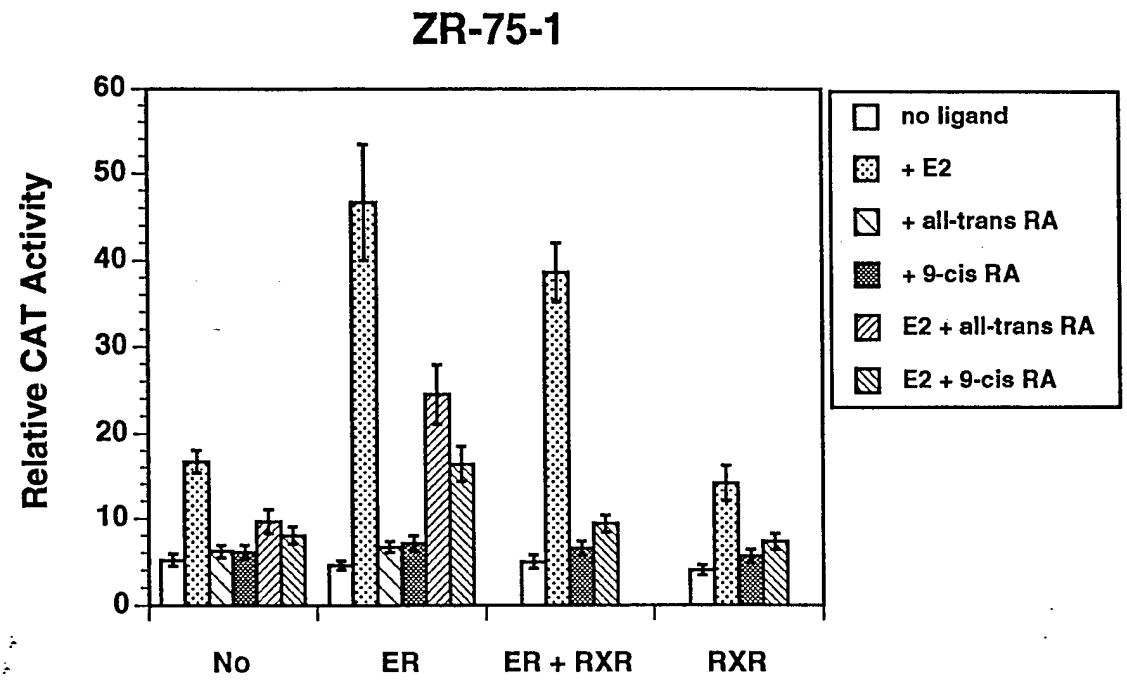

Figure 1. Repression of estrogen-induced activation of lactoferrin-RARE/ERE by RA in breast cancer cells. A. Sequence comparison of the RARE in the lactoferrin gene promoter and the RARE from the CRBPII gene promoter and a consensus ERE. Sequences that are closely related to the AGGTCA motif are indicated by arrows. B. Repression of estrogen-induced activation of lactoferrin-RARE/ERE by RA in ZR-75-1 breast cancer cells. Lactoferrin-RARE/ERE was cloned into pBLCAT2, and the resulting reporter construct, lactoferrin-RARE/ERE-tk-CAT, was transfected into ZR-75-1 cells together with or without expression vectors for ER (50 ng), RARα (50 ng) or RXRα (20 ng). Cells were treated with or without all-*trans* RA (10^{-7} M), 9-cis RA (10^{-7} M) or E2 (10^{-8} M), and 24 h later assayed for CAT activity. Data shown represent the means of three independent experiments.

Inhibition of AP-1 activity by RA in breast cancer cells.

Since cancer is a malignancy in which the balance between growth and differentiation is disturbed, the anti-cancer activity of retinoids is believed to be at least partially due to their direct anti-proliferative effects. This has been observed in a series of transformed cell lines, including mammary, melanoma, lymphoid and fibroblastic. The mitogenic stimuli, often generated by the autocrine secretion of growth factors is transmitted to the cell nucleus via certain second messenger pathways. Although starting out from different growth factors and the usage of distinct pathways, it is often the activation of the nuclear transcriptional factors cJun and cFos, the component of AP-1, that trigger cell proliferation. We have previously shown that RARs, in response to RA, can antagonize the activities of c-jun and c-fos. Such interactions between membrane and receptor pathways in nucleus may be essential for the control of cellular proliferation. To determine whether breast cancer cells contain TPA-induced AP-1 activities, -73Col-CAT, that contains collagenase promoter linked with CAT gene, was transfected into ZR-75-1 or T-47D cells. When cells were treated with TPA, we observed a strong induction of reporter activity, which was significantly inhibited when RA was present (Fig. 2). These results suggest that under certain conditions the anti-AP-1 activity of retinoids may contribute to the growth inhibitory effect of *trans*-RA in these breast cancer cells.

We then determined whether retinyl methyl ether (RME) that is a well known inhibitor of mammary cancer development (16,17) could exert its anti-cancer effect by downregulating AP-1 activity **(Agadir et al., Cancer Res. 57: 3444-3450, 1997)**. In this study, we investigate whether RME can down-regulate transcriptional activation by the tumor promoter TPA, growth factor and the nuclear protooncogenes cJun and

cFos. Transient transfection assays demonstrate that RME can repress transcriptional induction by the tumor promoter 12-0-tetradecanoylphorbol-13-acetate TPA, EGF, insulin, cJun and cFos of the human collagenase promoter or a heterologous promoter that contains the AP-1 binding site. In HeLa cells, the inhibition was observed when RARα expression vector was cotransfected, while the endogenous retinoid receptors in breast cancer cells are sufficient to confer the inhibition by RME. In addition, the expression of nur77 induced by TPA and growth factor could be inhibited by RME. These results suggest that inhibition of AP-1 responsive genes may represent an important mechanism by which RME prevents cancer development.

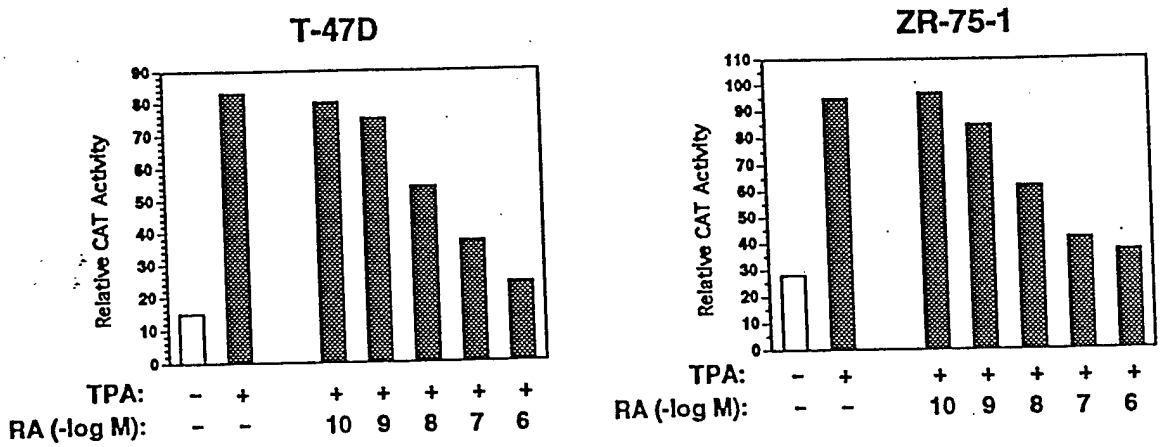

Figure 2. Inhibition of TPA-induced AP-1 activity in breast cancer cells by trans RA and retinoids. ZR-75-1 and T-47D cells were transiently transfected with 250 ng of -73ColCAT reporter gene. After transfection, cells were grown in 0.5% charcoal-treated FCS in the presence or absence of indicated amounts of *trans*-RA with or without TPA (100 ng/ml^{-1}) for 24 h. The β-gal activity was used to normalize for CAT activity. The assay were as described previously.

Induction of RARβ plays a critical role in mediating the growth inhibitory effect of retinoids in breast cancer cells

Retinoids are known to inhibit the growth of hormone-dependent but not of hormone-independent breast cancer cells. To establish the involvement of retinoid receptors in the differential growth inhibitory effect of RA on breast cancer cells, we examined the expression of three types of RAR (α, β, and γ) and of RXR (α, β and γ) in various hormone-dependent and -independent breast cancer cell lines (**Liu et al., Mol. Cell. Biol. 16: 1138-1149, 1996**). Transcripts for RARα, RARγ, RXRα, and RXRβ were detected in all the cell lines with minor variations in expression levels while the expression of RXRγ gene was not observed under the conditions used. When the expression of the RARβ gene was examined, we found that it was strongly enhanced by RA in hormone-dependent breast cancer cell lines, while treatment with RA failed to induce its expression in hormone-independent cell lines (Figure 3). In examining the growth inhibitory effect of all-trans RA and 9-cis RA, we observed that both RAs showed a strong inhibition of the growth of hormone-dependent cell lines while they had no effect on hormone-independent lines. Thus, the induction of RARβ gene expression by RA correlates to the growth inhibitory effect of RAs, suggesting that RARβ may mediate the RA effect. To directly test this, cDNA for the RARβ gene was stably expressed in hormone-independent breast cancer cells (MB231). When the anchorage-dependent and -independent growth of RARβ transfectants was measured in the presence

and absence of RA, we observed a strong growth inhibition of RARβ transfectant cells in the presence of RA. Thus, the expression of the RARβ gene can restore the growth inhibitory effect of RA in hormone-independent breast cancer cells. In addition, RA sensitivity of hormone-dependent cells was inhibited by a RARβ-selective antagonist and the expression of RARβ anti-sense RNA. Together, RARβ can mediate retinoid action in breast cancer cells and the loss of RARβ may contribute to the tumorigenicity of human mammary epithelial cells.

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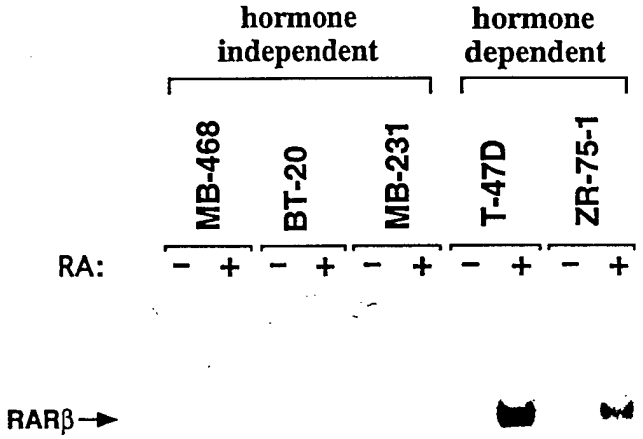

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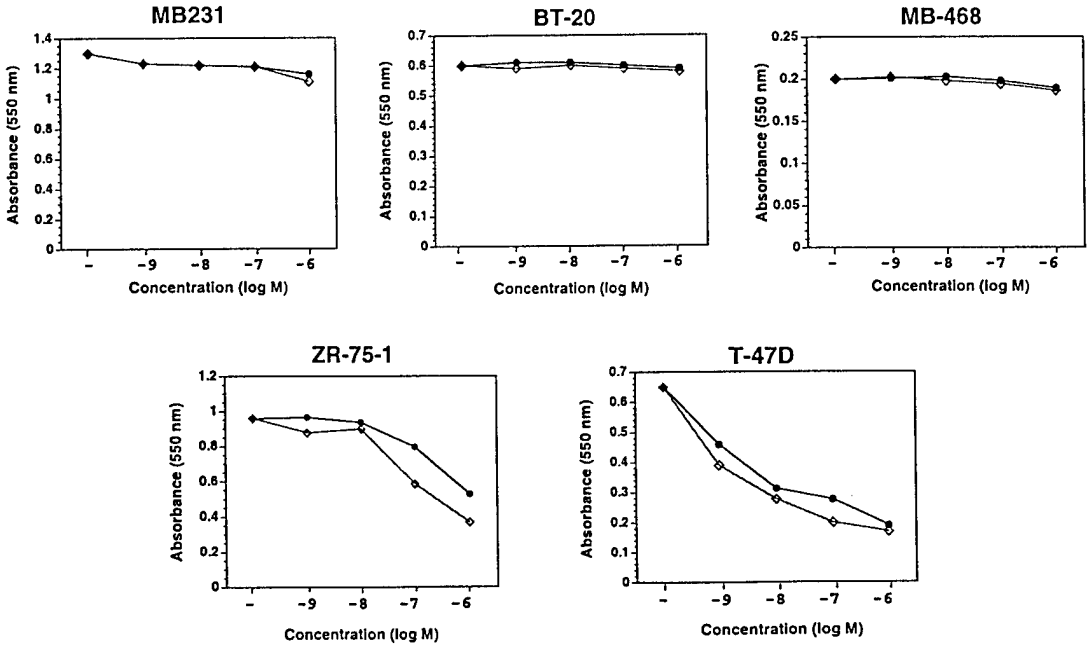

Figure 2. Expression of RARβ correlates with growth inhibitory effect of RA in human breast cancer cells. A. Expression of RARβ in hormone-dependent and -independent human breast cancer cell lines. The

expression of RARβ was determined by Northern blot analysis using total RNA (about 30 μg) prepared from different breast cancer cell lines treated with or without RA for 24 h. **B.** Effect of all-trans RA and 9-cis RA on the growth of hormone-dependent and -independent breast cancer cells. 2,000 cells per well were seeded and treated with various concentrations of RAs for 7 days.

RARβ promotes apoptosis in breast cancer cells

Apoptosis or programmed cell death is a fundamental important physiologic process in normal development and tissue homeostasis. Although the biochemical pathway that mediates apoptosis is still unknown, in many cases apoptosis is controlled by a number of factors including extracellular ligands such as steroids, growth factors, intracellular mediators of signal transduction, nuclear proteins regulating gene expression, DNA replication, and cell cycle. Recently, apoptosis has been recognized as another important pathway that helps restrict cell proliferation. Suppression of normal apoptosis can result in abnormal cell survival and malignant growth. Several studies have demonstrated that tumor cells can be eliminated by artificially triggering death through apoptosis. When RARβ was expressed in MB-231 cells, we noticed that many RA-treated cells detached and became shrunken, followed by cell death. When the nuclei of these cells were stained by propidium iodine (PI) and examined by fluorescence microscopy, we found that many of the RA-treated cells were smaller and contained fragmented nuclei with brightly staining chromatin, i.e., morphological changes typical of apoptosis. RA caused similar morphological changes in RA-sensitive ZR-75-1, MCF-7 and T-47D cells but not in the RA-resistant MB-231 and MB-468 cells. Furthermore, DNA fragmentation indicative of apoptosis was also detected in ZR-75-1 and MB-231 introduced with RARβ. DNA fragmentation was found in more than half of the RA-treated ZR-75-1 cells, and occurred as early as 12 hours after exposing cells to RA. Interestingly, ZR-75-1 cells that expressed RARβ anti-sense RNA experienced significantly less DNA fragmentation in response to RA (Figure 4). Thus, RA can induce apoptosis in RA-sensitive hormone-dependent breast cancer cells, which is likely mediated by RARβ. Together, retinoids may suppress breast cancer cell growth by the process of apoptosis.

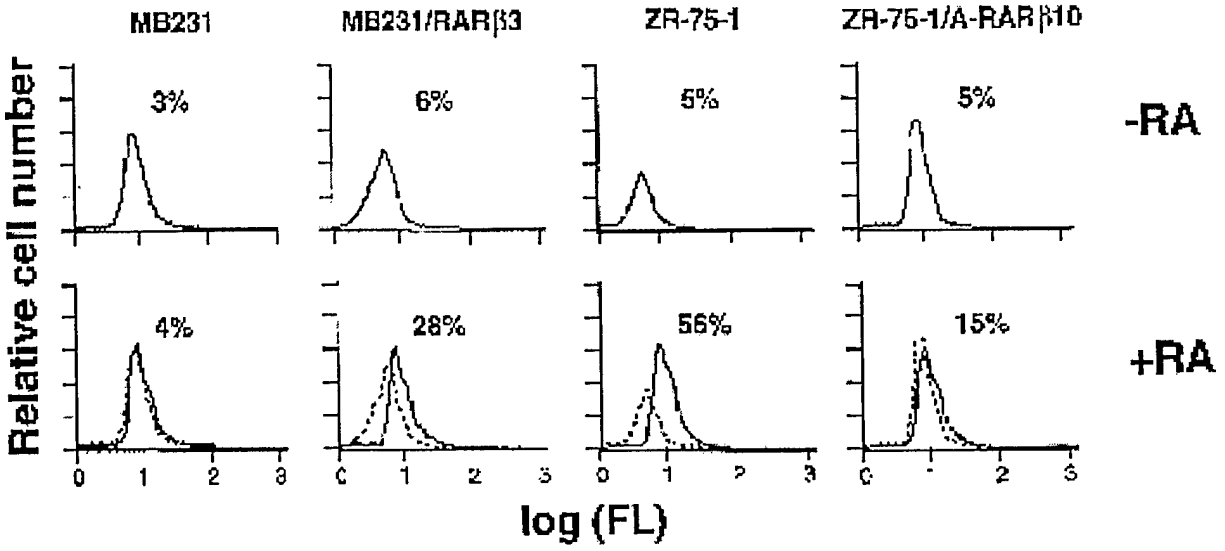

Figure 4. RARβ promotes cell apoptosis. TUNEL assays of parental and RARβ transfected MB231 (MB231/RARβ3) cells, and parental and antisense RARβ transfected ZR-75-1 (ZR-75-1/A-RARβ10) cells after 24 hours of RA treatment.

Interaction of BAG-1 with RAR regulates RA-induced apoptosis in breast cancer cells

BAG-1 (also known as RAP46) is an anti-apoptotic protein. It was cloned from a murine embryo cDNA library using a protein-protein interaction technique (19). Co-expression of BAG-1 and Bcl-2 in Jurkat lymphoid cells, NIH 3T3 fibroblasts, and melanoma cells promoted the survival of these cells in response to a variety of apoptotic stimuli (19). In addition to Bcl-2, BAG-1 also interacts with Raf-1 (20), and can activate this kinase through a Ras-independent mechanism. Furthermore, BAG-1 can interact with hepatocyte growth factor (HGF) receptor and with platelet-derived growth factor (PDGF) receptor, and enhance the ability of these receptors to transduce signals for cell survival (21). These observations suggest that BAG-1 may function as an adaptor to mediate the interaction between survival factors and apoptotic machinery, and may also play a role in regulating cellular proliferation. Interestingly, the human BAG-1 homolog (also known as RAP46) was cloned from a human liver cDNA library by virtue of its interaction with the glucocorticoid receptor (22). In vitro, RAP46 interacts with a number of nuclear hormone receptors, including estrogen receptor (ER) and thyroid hormone receptor (TR) (22). Recently, it has been shown that BAG-1 binds tightly to Hsp70/Hsc70-family proteins and modulates their chaperone activity (23). Since molecular chaperones are known to play an important role in controlling the activity of many members of the steroid/thyroid/retinoid receptor family (24), it is possible that BAG-1 could alter the function of these transcriptional regulators. Prior to this report, however, it was unknown whether BAG-1 regulates the activities of the nuclear hormone receptors and whether BAG-1 interacts with retinoid receptors. We studied whether BAG-1 interacts with RAR. Gel retardation assays demonstrated that in vitro translated BAG-1 protein could effectively inhibit the binding of RAR/RXR to the βRARE (**Liu et al. J. Biol. Chem. 273: 16985-16992, 1998**). A glutathione S-transferase (GST)-BAG-1 fusion protein also specifically bound RAR but not RXR (Figure 5). Interaction of BAG-1 and RAR could also be demonstrated by yeast two-hybrid assays. In transient transfection assays, co-transfection of BAG-1 expression plasmid inhibited the transactivation activity of RAR/RXR heterodimers (Figure 4). When stably expressed in both MCF-7 and ZR-75-1 breast cancer cell lines, BAG-1 suppressed retinoic acid (RA)-induced growth inhibition and apoptosis. In addition, RA-induced suppression of Bcl-2 expression was abrogated by over-expression of BAG-1. Taken together, our results demonstrate that BAG-1 can physically interact with RARs and is an important component of the retinoid response pathway. The findings suggest that this protein-protein interaction may play an important role in the regulation of retinoid-induced growth inhibition and apoptotic processes, potentially contributing to retinoid resistance in cancer.

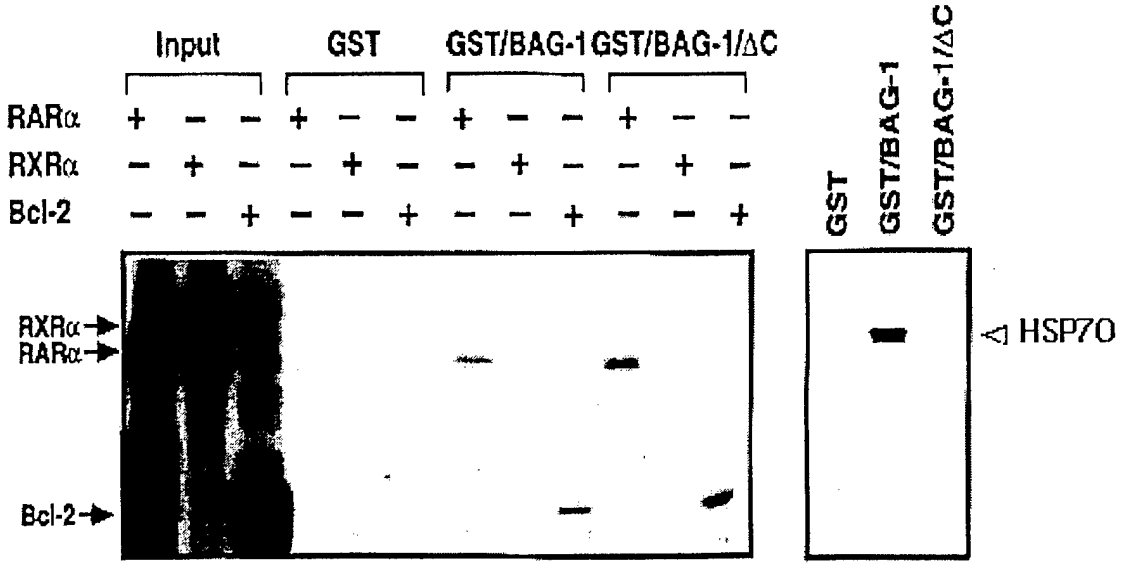

Figure 5. Analysis of RAR-BAG-1 interaction by the GST pull-down assay. BAG-1 or a BAG-1 C-terminal deletion mutant (BAG-1/Δc) was expressed in bacteria using the pGex.4T expression vector. The GST-BAG-1 proteins were immobilized on glutathione-Sepharose beads. As a control, the same amount of GST was also immobilized. ^{35}S-labeled RARα, RXRα or Bcl-2 was then mixed with the beads. After extensive washing, the bound proteins were analyzed by SDS-PAGE. The input proteins are shown for comparison (left panel). For comparison, binding of GST-BAG-1 and GST-BAG-1/Δc to Hsc70 was shown in right panel.

A RXR-dependent pathway induces RARβ expression and growth inhibition in breast cancer cells

We showed previously that activation of RARs, but not RXRs is required for growth inhibition in estrogen-dependent ZR-75-1 cells (13). To further investigate the role of RARs and RXRs in breast cancer cells, we used a number of RAR-selective and RXR-selective retinoids to determine their growth inhibitory effects in both *trans*-RA-sensitive, estrogen-dependent and *trans*-RA-resistant, estrogen-independent cells. RAR-selective retinoids strongly inhibited the growth of *trans*-RA-sensitive ZR-75-1 cells, but not *trans*-RA-resistant cells. In contrast, RXR-selective retinoids did not affect *trans*-RA-sensitive cell growth, but significantly inhibited the growth of *trans*-RA resistant MDA-MB-231 cells. RXR-selective retinoid also induced apoptosis in *trans*-RA-resistant cells but not in *trans*-RA-sensitive cells, whereas RAR-selective but not RXR-selective retinoid induced apoptosis only in ZR-75-1 cells **(Wu et al., Mol. Cell. Biol. 17: 6598-6608, 1997)**. These data suggest that the RXR pathway is involved in *trans*-RA-resistant breast cancer cell growth inhibition and apoptosis. The combination of either RXR-selective retinoid with the RAR-selective retinoid strongly enhanced the growth inhibitory effect of the RXR-selective retinoids. Thus, different retinoid signaling pathways can mediate retinoid-induced growth inhibition in estrogen-dependent and -independent breast cancer cells.

We showed previously that expression of RARβ can mediates the growth inhibitory effects of RA (13). To determine whether the growth inhibitory effects of RXR-selective retinoids are also mediated by expression of RARβ, we analyzed RARβ gene expression in both estrogen-dependent and -independent breast cancer cell lines in response to RAR-selective and RXR-selective retinoids. RAR-selective retinoids strongly induced RARβ expression in *trans*-RA-sensitive ZR-75-1 and T-47D cells, while RXR-selective retinoids did not. In contrast, both RAR-selective and RXR-selective retinoids could slightly induce RARβ in *trans*-RA-resistant MDA-MB-231 cells. The combination of RAR-selective and RXR-selective retinoids induced RARβ to a level comparable to that observed in T-47D cells. Thus, our data demonstrate that RARβ induction and activation correlates with growth inhibition and suggest that RARβ induction may contribute to growth inhibition by RXR-selective retinoids.

The βRARE in the RARβ gene promoter mediates RARβ expression (10). The βRARE is activated by the RXR/RAR heterodimer in response to RAR-selective retinoids, but not RXR-selective retinoids (8). Recently, orphan receptor nur77 was shown to bind to DR-5 type RARE as RXR/nur77 heterodimers (8, 9). We investigated the binding of nur77 on the βRARE. Nur77 alone did not show clear binding, but when mixed with RXRα, strong binding to βRARE occurred (14,15). Thus, the βRARE is an unique RARE that allows interaction with both RXR/nur77 and RXR/RAR heterodimers. To determine whether binding of the RXR/nur77 heterodimers to the βRARE could activate the response element in response to RXR-selective retinoids, we performed a transient transfection assay (15). Cotransfection of nur77 and RXR expression vectors significantly enhance the βRARE activity in response to RXR-selective retinoids. This data suggests that induction of RARβ by RXR-selective retinoids through RXR/nur77 heterodimers may be the mechanism by which RXR-selective retinoids inhibit the growth of MDA-MB-231 cells.

Many estrogen-dependent and -independent breast cancer cell lines have similar expression levels of RARγ and RXRα (13). Nur77 expression levels are similar in ZR-75-1 and MDA-MB-231 cells. However, RARα is only highly expressed in the estrogen-dependent, *trans*-RA-sensitive breast cancer cell lines (15). This suggests the possibility that expression of RARα may allow preferential formation of RARα/RXR heterodimers in estrogen-dependent breast cancer cell lines, that function to mediate the growth inhibitory effect of RAR-selective retinoids but not RXR-selective retinoids. In contrast, low expression level of RARα in estrogen-independent, *trans*-RA-resistant breast cancer cell

lines may permit formation RXR/nur77 heterodimers that can be activated by RXR-selective retinoids to induce RARβ. When we stably express RARα in MDA-MB-231 cells to enhance the level of RARα, the effects of RAR-selective retinoids on growth inhibition and RARβ induction was enhanced while the effect of RXR-selective retinoids was suppressed. The low RARα level in MDA-MB-231 cells (13) favors nur77/RXR formation that activates the βRARE in response to RXR-selective retinoids. Therefore, depending on the RAR, RXR, and nur77 levels present in the cancer cells, either the *trans*-RA- or 9-*cis* RA-signaling pathway can activate the βRARE (Figure 6). This retinoid signaling switch may play an important role in regulating cell growth in response to different stimuli and suggests that low expression of RARα may allow 9-*cis*-RA signaling but cause *trans*-RA resistance.

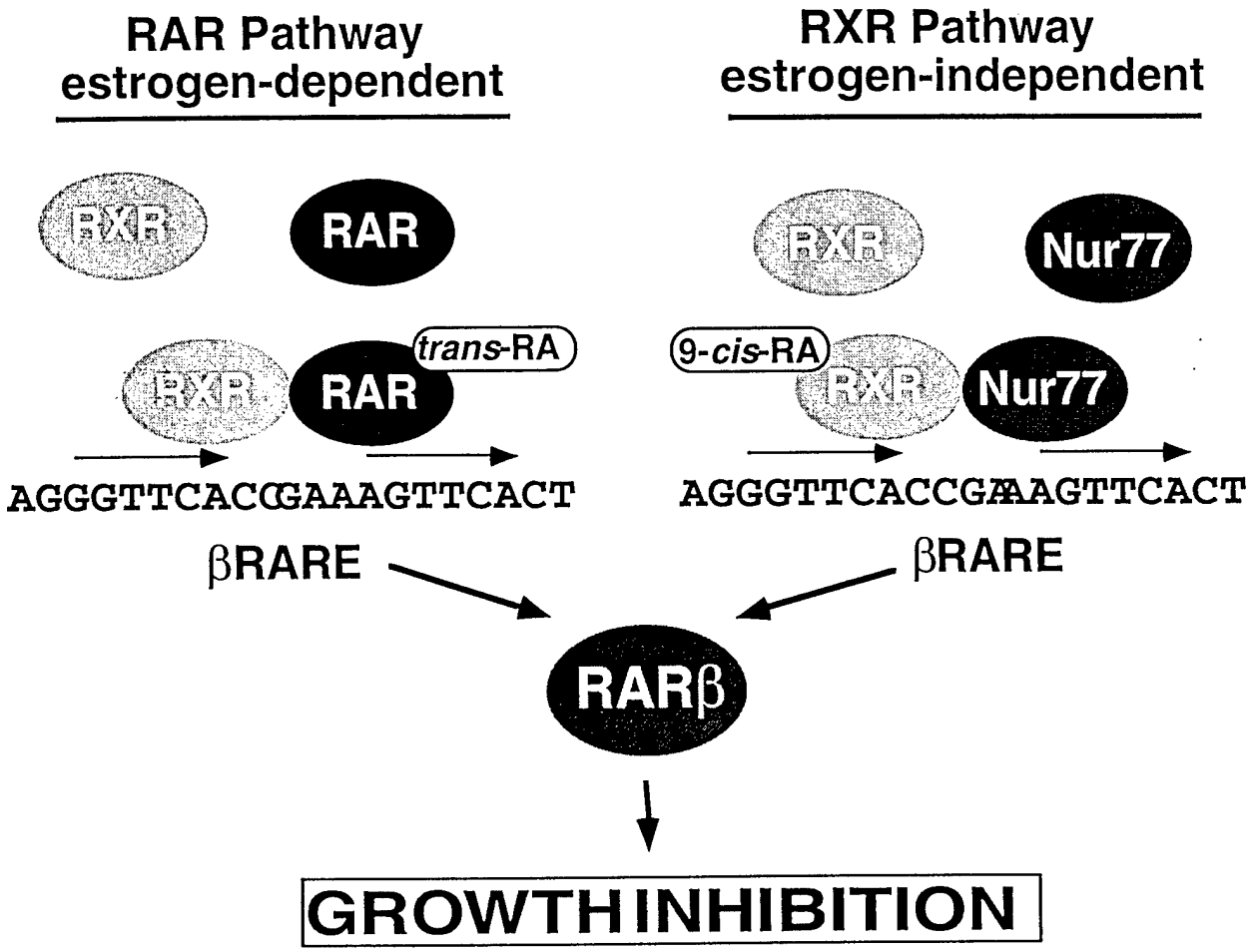

Figure 6. Retinoid signaling pathways in breast cancer cells. RARβ expression can mediate the growth inhibitory effect of retinoids and is regulated by the βRARE in RARβ promoter. βRARE bound to both RXRα/RARα and RXRα/nur77. Relative cellular levels of RAR, RXR and nur77 may determine whether βRARE binds to RXRα/RARα or RXRα/nur77. Estrogen-dependent, *trans*-RA-sensitive breast cancer cells expressed higher levels of RARα to favor binding of RXRα/RARα that can be activated by RAR-selective ligands to induce RARβ expression. In contrast, estrogen-independent, *trans*-RA-resistant breast cancer cells expressed lower levels of RARα to favor RXRα/nur77 formation, which bound to the βRARE. RXR-selective retinoids activate nur77/RXR to induce RARβ expression. Induction of RARβ by either RAR- or RXR-selective retinoids could lead to growth inhibition.

Orphan receptor COUP-TF and RA-induced RARβ expression, growth inhibition and apoptosis in breast cancer cells.

COUP-TF is required for RAR β induction, growth inhibition and apoptosis induction by RA in breast cancer cells. In addition to retinoid receptors, a number of orphan receptors whose ligands are unknown have been implicated in the regulation of retinoid response. One of the factors is COUP-TF. COUP-TF is encoded by two distinct genes, COUP-TFI (ear-3) and COUP-TFII (ARP-1). COUP-TF was originally shown to stimulate gene transcription. However, subsequent work has demonstrated that COUP-TF can repress transcription induced by a number of nuclear receptors including RARs, thyroid hormone receptors, and vitamin D receptor (4,5). To determine whether expression of COUP-TF is involved inhibition of RARβ expression, we evaluated expression of COUP-TF in various RARb-positive and –negative breast cancer cell lines (**Lin et al., Mol. Cell. Biol. In press, 2000**). Our data demonstrated a perfect correlation between COUP-TFI expression and the ability of RA to induce RARβ in breast cancer cell lines (Figure 7). COUP-TFI was expressed in ZR-75-1 and T-47D cell lines, in which RARβ expression was highly induced by RA. In contrast, COUP-TF transcripts were not detected in MDA-MB468 and MDA-MB231 cell lines that did not show a clear induction of RARβ by RA. These observations suggest that expression of COUP-TF may be required for RA to induce RARβ expression in different types of cancer cells.

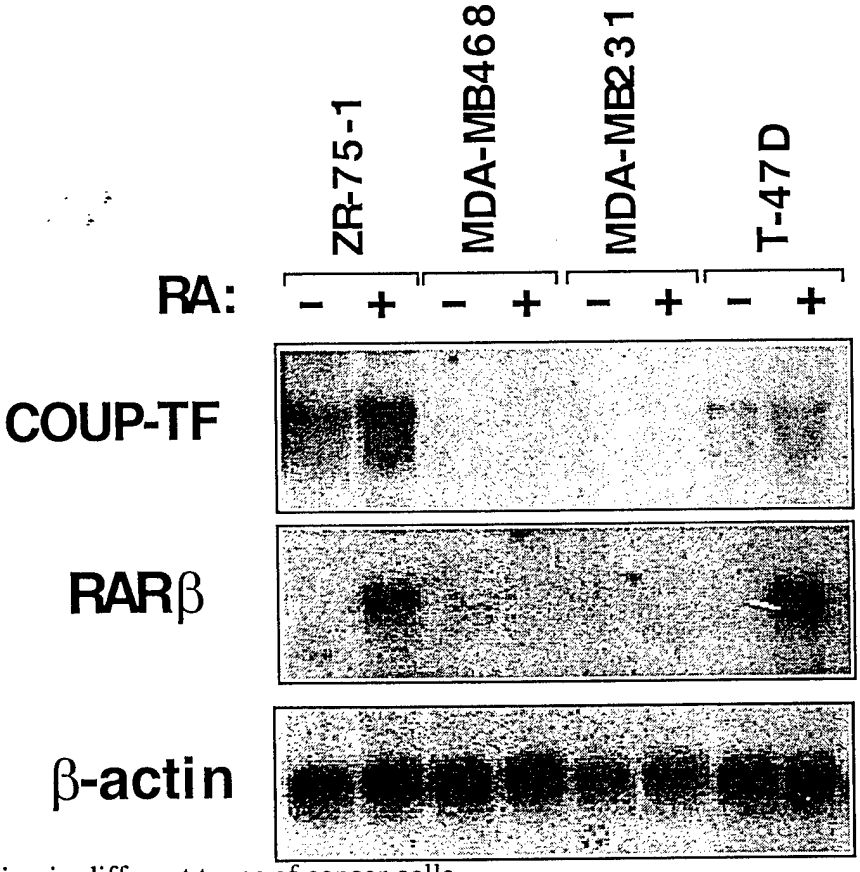

Figure 7. Correlation between COUP-TF expression and RARβ induction by RA in human breast cancer cell lines. Total RNAs were prepared from the indicated cancer cell lines treated with or without 10^{-6} M all-*trans* RA for 24 h and analyzed for the expression of COUP-TFI and RARβ. For control, the expression of β-actin is shown.

To further determine the requirement of COUP-TF for RA-dependent activation of RARβ gene expression, we stably expressed COUP-TFI in COUP-TF-negative MDA-MB231 breast cancer cells. Stable clones that expressed a high level of transfected COUP-TFI were subject to analysis of RARβ gene expression in the absence or presence of RA. RA-treatment strongly induced RARβ expression in the COUP-TFI-stable clones, but not in the MDA-MB231 cells transfected with the empty vector (MB231/vector) (**Lin et al., Mol. Cell. Biol. In press, 2000**). This suggests that expression of COUP-TFI confers MDA-MB231 cells sensitivity to RA regulation of RARβ expression. When the effect of COUP-TFI stable transfection on growth of MDA-MB231 cells was analyzed, we observed that RA, that was unable to regulate the growth of the parental MDA-MB231 cells, could strongly inhibit the growth of the COUP-TFI stable clones. The TdT assay also showed extensive DNA fragmentation in COUP-TF stable clones, but not in the parental MDA-MB231 cells. Thus, expression of COUP-TFI in COUP-TF-negative breast cancer cells can restore their response to RA effects on RARβ expression, growth inhibition and apoptosis.

COUP-TF enhances RARβ promoter activity in a RARα- and RA-dependent manner.

To investigate the possibility that COUP-TFI induced RARβ expression by activating RARβ promoter transcription, a reporter construct that contains the RARβ promoter was transiently transfected into CV-1 cells. Cotransfection of RARα expression vector induced RARβ promoter activity in response to RA (Figure 8), while cotransfection of COUP-TFII expression vector slightly enhanced the reporter gene activity. However, when both COUP-TFII and RARα were cotransfected, a synergistic induction of RARβ promoter activity in response to RA was observed (Figure 8). Together, our data demonstrate that expression of COUP-TF is required for efficient induction of RARβ promoter activity by RA in a RARα-dependent manner.

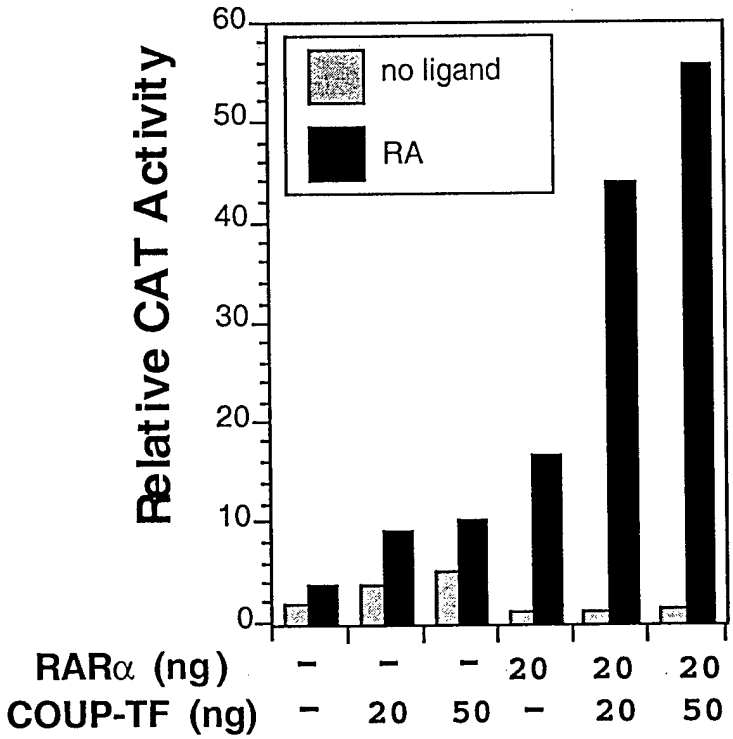

Figure 8. COUP-TF enhances RARβ promoter activity in a RARα and RA-dependent manner. A. Activation of RARβ promoter activity by COUP-TF in CV-1 cells. 700 ng RARβ promoter reporter (-745RARβCAT) was co-transfected with the indicated amount of expression vector for COUP-TFII

and RARα into CV-1 cells. Cells were treated with (black bar) or without (grey bar) 10^{-6} M all-*trans* RA for 24 h and assayed for CAT activity. Data shown represent the means of three independent experiments.

A DR-8 element in the RARβ promoter mediates COUP-TF effect.

To identify DNA sequences responsible for COUP-TF effect in the RARβ promoter, we generated a series of 5' deletion mutants of the RARβ promoter. The resulting RARβ promoter fragments were fused to the CAT reporter gene and analyzed for effect of COUP-TFII on enhancing RA-induced RARα activity in CV-1 cells. Our mutational analysis revealed that a direct repeat of AGGTCA-like motifs with 8 bp spacing (DR-8), located from –99 to –78 of the RARβ promoter, is required for COUP-TF to enhance RARβ promoter activity. The COUP-TF response element (COUP-TF-RE) could bind strongly with COUP-TF protein (Figure 9). By using a variety of approaches, we demonstrated that the COUP-TF-RE is essential but not sufficient to confer the enhancing effect of COUP-TF in the RARβ promoter (**Lin et al., Mol. Cell. Biol. In press, 2000**). We therefore analyzed the involvement of βRARE by mutational analysis since it is the only known sequence in the RARβ promoter that mediates RA effect. Our data demonstrated that intact of the βRARE, capable of binding with RARα/RXRα heterodimer, is essential for transactivation function of COUP-TF. Thus, both βRARE and DR-8 element are required for RA- and RARα-dependent transactivational function of COUP-TF in the RARβ promoter.

COUP-TF enhances recruitment of CBP by RARα

Transactivation function of nuclear receptors requires their interaction with receptor co-activators, such as CBP. The transactivation function of COUP-TF could be due to its interaction with CBP. We, therefore, investigated whether COUP-TFI could interact with CBP by the GST-pull-down assay. Under conditions used, we did not observe a clear interaction between COUP-TFI and CBP. This demonstrates that the transactivation function of COUP-TFI is unlikely mediated through its direct interaction with CBP. The facts that the transactivation function of COUP-TF is RARα- and RA-dependent and requires βRARE suggest that effect of COUP-TF may be mediated by RARα that is known to interact with CBP. Indeed, our result showed that both RARα and COUP-TFI are required for their maximum interaction with CBP (**Lin et al., Mol. Cell. Biol. In press, 2000**). Thus, COUP-TF may facilitate RARα/CBP interaction by interacting with RARα, resulting in a RARα conformation favorable for CBP interaction. Thus, COUP-TF induces RARβ promoter transcription by acting as an accessory protein for RARα to recruit its co-activator (Figure 9).

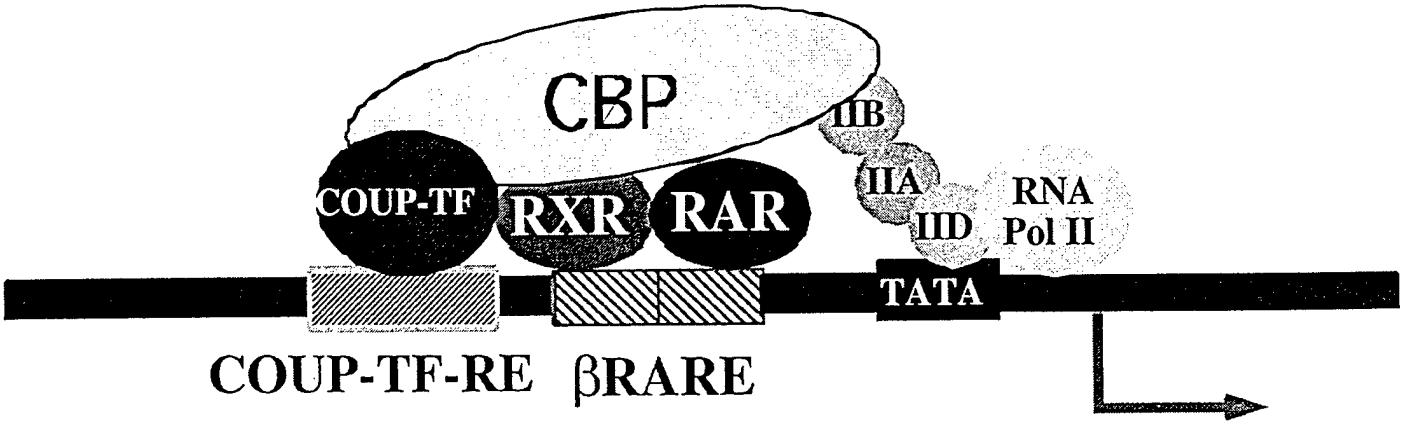

Figure 9. Model of COUP-TF action in the RARβ promoter.

CONCLUSION

Through the funding of this project, we have made substantial progress towards understanding molecular mechanism of retinoid action in breast cancer cells, and have accomplished most aims proposed.

We demonstrated that retinoids exerts their effects in part through their interference of mitogenic effects of estrogen receptor. In addition, we showed that they could also inhibit AP-1 activity. Furthermore, we discovered that retinoids could inhibit growth of breast cancer cells through induction of apoptosis. Thus, different mechanisms may contribute to the growth inhibition of breast cancer cells depending on cell type or growth conditions.

Our results convincingly demonstrated that expression of RARβ plays a critical role in mediating the growth inhibitory effects of RA in both estrogen-dependent and -independent breast cancer cell lines. We also showed for the first time that RARβ acts to induce apoptosis, providing a new mechanism by which retinoid receptors exert their anti-cancer activity. In addition, we reported that retiniod receptors could interact with a cell survival gene, BAG-1, providing a mechanism for the interaction between retinoid and apoptosis signalings.

We also demonstrated that loss of retinoid activity in certain malignant hormone-independent breast cancer cells is due to a loss of RARβ. We showed recently that loss of RARβ is mainly due to lack of orphan receptor COUP-TF that acts through a new mechanism to induce RARβ in response to RA. These discoveries provide important directions to restore retinoid activity in malignant retinoid-resistant breast cancer cells and to develop more effective retinoids against breast cancer.

Our results revealed a new retinoid pathway that induces growth inhibition in estrogen-independent, *trans*-RA-resistant breast cancer cells. The new pathway is mediated through RXR/nur77 heterodimers that induce RARβ gene expression in response to RXR ligands. This finding is important in that the pathway is functional in RARα-negative, estrogen-independent and *trans*-RA-resistant breast cancer cells. Thus, different retinoid growth inhibition pathways exist and different classes of retinoids can be used for different types of breast cancer. This finding provide a molecular basis to develop new retinoids for different types of breast cancer cells.

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14. Identification of a Novel Class of RARβ-selective Retinoid Antagonists and their Inhibitory Effects on AP-1 activity and Retinoic Acid-induced Apoptosis in Human Breast Cancer Cells. (Li, Y., , Hashimoto, Y, Agadir, A., Kagechika, H., and Zhang, X.-k., **J. Biol. Chem**. 274: 15360-15366, 1999)

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APPENDIX

1. Inhibition of *trans*-RA-resistant human breast cancer cell growth by retinoid X-receptor-selective retinoids. (Wu, Q., Cheng, Y., Dawson, M.I., Hobbs, P.D., Li, Y., and Zhang, X.-k. **Mol. Cell. Biol.** 17: 6598-6608, 1997).

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Inhibition of *trans*-Retinoic Acid-Resistant Human Breast Cancer Cell Growth by Retinoid X Receptor-Selective Retinoids

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All-*trans*-retinoic acid (*trans*-RA) and other retinoids exert anticancer effects through two types of retinoid receptors, the RA receptors (RARs) and retinoid X receptors (RXRs). Previous studies demonstrated that the growth-inhibitory effects of *trans*-RA and related retinoids are impaired in certain estrogen-independent breast cancer cell lines due to their lower levels of RARα and RARβ. In this study, we evaluated several synthetic retinoids for their ability to induce growth inhibition and apoptosis in both *trans*-RA-sensitive and *trans*-RA-resistant breast cancer cell lines. Our results demonstrate that RXR-selective retinoids, particularly in combination with RAR-selective retinoids, could significantly induce RARβ and inhibit the growth and induce the apoptosis of *trans*-RA-resistant, RARα-deficient MDA-MB-231 cells but had low activity against *trans*-RA-sensitive ZR-75-1 cells that express high levels of RARα. Using gel retardation and transient transfection assays, we found that the effects of RXR-selective retinoids on MDA-MB-231 cells were most likely mediated by RXR-nur77 heterodimers that bound to the RA response element in the RARβ promoter and activated the RARβ promoter in response to RXR-selective retinoids. In contrast, growth inhibition by RAR-selective retinoids in *trans*-RA-sensitive, RARα-expressing cells most probably occurred through RXR-RARα heterodimers that also bound to and activated the RARβ promoter. In MDA-MB-231 clones stably expressing RARα, both RARβ induction and growth inhibition by RXR-selective retinoids were suppressed, while the effects of RAR-selective retinoids were enhanced. Together, our results demonstrate that activation of RXR can inhibit the growth of *trans*-RA-resistant MDA-MB-231 breast cancer cells and suggest that low cellular RARα may regulate the signaling switch from RAR-mediated to RXR-mediated growth inhibition in breast cancer cells.

Retinoids, the natural and synthetic vitamin A analogs, exert profound effects on cell proliferation, differentiation, and apoptosis (19, 36, 50) and are considered promising agents for the prevention and treatment of human cancers, including breast cancer (36, 43, 50). Retinoids, alone or in combination with an antiestrogen or interferons, inhibit the in vitro growth of human breast cancer cells (12–14, 29, 34, 51, 60–62). The natural retinoid derivative retinyl methyl ether (18) and the synthetic retinoids *N*-(4-hydroxyphenyl) retinamide (4-HPR) (44, 45) and LGD1069 (17) effectively inhibited the development of carcinogen-induced mammary cancers in animals. Unfortunately, clinical trials on patients with advanced breast cancer showed no significant activity for retinoids (2, 3, 42). These studies indicate that retinoids are effective inhibitors of the cancer cells at the early stages of tumor progression and that their effectiveness diminishes as cells become more malignant and invasive. They are also consistent with well-documented in vitro observations that growth inhibition by all-*trans*-retinoic acid (*trans*-RA) and related retinoids occurs mainly in estrogen-dependent, estrogen receptor-positive breast cancer cells and that upon progression to estrogen independence and loss of the estrogen receptor, most breast cancer cells become refractory to growth inhibition by *trans*-RA (14, 34, 54, 55, 60).

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors (RARs) and retinoid X receptors (RXRs) (28, 38, 70). 9-*cis*-RA is a high-affinity natural ligand for both RARs and RXRs, whereas *trans*-RA is a high-affinity natural ligand only for the RARs. RARs and RXRs are each encoded by three distinct genes (α, β, and γ) and are members of the steroid/thyroid hormone/retinoid receptor superfamily, which function as ligand-activated transcription factors (28, 38, 70). RARs interact with RXRs, forming RXR-RAR heterodimers that bind to RA response elements (RAREs) to control the expression of RA-responsive genes in the presence of retinoids. Transcriptional regulation of RA-responsive genes is also modulated by a number of cofactors that appear to provide a direct link to the core transcriptional machinery and/or to modulate chromatin structure (reference 27 and references therein). Although RXR acts as a silent heterodimerization partner of RAR in CV-1 cells (15, 30, 38), recent studies demonstrate that binding of certain RXR ligands contributes to activation of RXR-RAR heterodimers in some cell types (4, 31, 40, 52, 59, 71). In the presence of 9-*cis*-RA, RXRs can also function as homodimers that bind a set of specific DNA sequences (68, 70, 71). Furthermore, activation of RXR is required for the function of other RXR-containing heterodimers, such as RXR-nur77 (15, 49) and RXR-LXR (63). Thus, distinct retinoid signaling pathways through activation of either RAR or RXR exist; however, the role of RXR activation in these pathways requires clarification.

RA target genes, including those for the RARs, have been identified. The RARE (βRARE) in the RARβ gene promoter mediates *trans*-RA-induced RARβ gene expression in many different cell types (10, 21, 57) and binds both RXR-RAR (28, 38, 70) and RXR-nur77 (15, 49) heterodimers. Gene transcriptional activation by RXR-RAR binding is mainly activated by RAR-specific ligands, while transactivation by RXR-nur77 is induced by RXR-specific ligands (15, 49). These observations

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suggest that RARβ can be induced by both RAR- and RXR-specific ligands. nur77 is an orphan member of nuclear receptor superfamily that regulates gene expression by binding to the nur77-binding response element (NBRE) as a monomer (64). It is rapidly induced by various stimuli, including growth factors and phorbol ester- and cyclic AMP-dependent synthesis pathways (20, 39). Recent studies suggest that nur77 is involved in activation-induced apoptosis of T cells (35, 65) and is associated with *trans*-RA resistance in human lung cancer cells (66). Thus, RARβ expression is regulated by growth signals and may be associated with the apoptotic process.

Recently, evidence has emerged that the absence or aberrant expression of RARβ correlates with malignancy and may contribute to the development of cancers. The involvement of RARβ in cancer development was originally implicated in the finding that the RARβ gene is integrated by hepatitis B virus in human liver cancer (9). Subsequent reports indicate that abnormal expression of the RARβ gene appears to be involved in the tumorigenicity of human papillomavirus type 18-transformed ovarian cancer cells (1) and the neoplastic progression of human oral squamous cell carcinoma cell lines (24), and it is observed in many other human cancer cell lines (16, 22, 24, 34, 48, 58, 69). RARβ also suppresses the growth of breast cancer cells (33, 34, 58) and lung cancer cells (23). The autoinduction of RARβ gene expression presumably plays a critical role in amplifying retinoid responses and is associated with the growth-inhibitory effects of *trans*-RA in breast cancer cells in vitro (34) and the clinical response to retinoids in patients with premalignant oral lesions (37). Retinoids that fail to induce RARβ expression cannot arrest the growth of melanoma cells (5).

The involvement of retinoid receptors in mediating retinoid-induced growth inhibition and apoptosis has been investigated. Several studies demonstrated that expression of RARα mediates the growth-inhibitory effect of *trans*-RA in estrogen-dependent breast cancer cells and that the loss of *trans*-RA sensitivity in estrogen-independent cells may be due to low levels of RARα (34, 51, 55, 60). RARα levels are higher in certain estrogen-dependent, *trans*-RA-sensitive breast cancer cell lines, such as ZR-75-1, MCF-7, and T-47D, than in certain estrogen-independent, *trans*-RA-resistant cells lines, such as MDA-MB-231 and MDA-MB-468 (34, 51, 55, 60). Expression of RARα in estrogen-independent, *trans*-RA-resistant MDA-MB-231 breast cancer cells restored *trans*-RA sensitivity (34, 54, 55, 61). Growth inhibition induced by retinoids in estrogen-dependent MCF-7 breast cancer cells correlated with their binding affinity to RARα (7). The involvement of RARβ was suggested by the observation that it was expressed in response to *trans*-RA in certain estrogen-dependent, *trans*-RA-sensitive breast cancer cell lines, such as ZR-75-1 and T-47D, but not in estrogen-independent, *trans*-RA-resistant cell lines MDA-MB-231, MDA-MB-468, and BT-20 (34). In addition, we (34) and others (33, 53) demonstrated that introduction of RARβ into MDA-MB-231 cells led to the recovery of *trans*-RA-induced growth inhibition. Moreover, RARβ expression was enhanced in RARα stably transfected MDA-MB-231 cells, a finding that suggests that RARβ may mediate the growth inhibitory effects of RARα (34). The role of RARβ in growth inhibition is also supported by the observation that as normal human mammary epithelial cells senesce, RARβ mRNA expression increases (58). RARγ is highly expressed in various breast cancer cell lines independently of their estrogen responsiveness (34) and is unlikely involved in regulating *trans*-RA-induced growth inhibition and apoptosis. However, recent studies (11, 12) have demonstrated that it may play a role in mediating growth inhibition and apoptosis induction by 4-HPR and certain synthetic retinoids. 4-HPR was a potent transactivator of RARγ at concentrations that inhibited the growth and induced apoptosis of breast cancer cells (11). Furthermore, growth inhibition by certain receptor-selective retinoids and interferons was associated with increased expression of RARγ (12). Thus, different retinoid receptors, which may function through different mechanisms, can mediate growth inhibition and apoptosis induction by different types of retinoids in breast cancer cells.

In this study, we evaluated the effects of RAR- and RXR-class selective retinoids on the growth of *trans*-RA-resistant, RARα-deficient MDA-MB-231 cells. Our results demonstrate that RXR-selective retinoids induced RARβ expression, growth inhibition, and apoptosis in these cells, most likely through their activation of RXR-nur77 heterodimers that bind to the RARβ promoter. When we stably expressed RARα in MDA-MB-231 cells, we observed an enhanced growth inhibition and RARβ induction by RAR-selective retinoids and decreased effects by RXR-selective retinoids, similar to those observed in *trans*-RA-sensitive, RARα-expressing breast cancer cells, such as ZR-75-1 cells. Thus, an RXR-mediated growth inhibition pathway exists in breast cancer cells and is regulated by RARα levels. These results may provide a novel method for inhibiting the growth of the more malignant *trans*-RA-resistant breast cancer cells.

MATERIALS AND METHODS

Retinoids. *trans*-RA was obtained from Sigma (St. Louis, Mo.). SR11246, SR11237, and SR11235 were prepared as described by Dawson et al. (8). Synthesis of SR11383 was described elsewhere (16a).

(*E*)-3-[4-(1-Methoxy-5,6,7,8-tetrahydro-1-5,5,8,8-tetramethyl-3-naphthalenyl) phenyl]propenoic acid (SR11278) was synthesized as follows. (i) Cyclialkylation [$AlCl_3$, $(CH_2Cl)_2$, 0°C] of 3-bromoanisole with 2,5-dichloro-2,5-dimethylhexane as reported by Kagechika et al. (26) yielded 3-bromo-1-methoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene (71%), which was coupled under Suzuki conditions {$Pd[P(C_6H_5)_3]_4$, $NaHCO_3$, aqueous 1,2-dimethoxyethane [MeO$(CH_2)_2$OMe], reflux} (41) with 4-formylphenylboronic acid to give 4-(1-methoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-3-naphthalenyl)benzaldehyde (91%). (ii) Horner-Emmons olefination of this benzaldehyde with triethyl phosphonoacetate [$KN(SiMe_3)_2$, tetrahydrofuran-toluene, −78 to 25°C] produced the ethyl ester of SR11278 (97%). (iii) Hydrolysis (KOH, aqueous ethanol [EtOH]; aqueous HCl) gave SR11278 (99%): melting point (mp), 179 to 182°C; ^1H nuclear magnetic resonance (NMR) (300 MHz, $^2HCCl_3$) δ 1.34 (s, 6, CMe_2), 1.41 (s, 6, CMe_2), 1.68 (m, 4, CH_2CH_2), 3.89 (s, 3, OMe), 6.49 (d, J = 16.0 Hz, 1, $C{=}CHCO_2$), 6.90 (d, J = 1.8 Hz, 1, ArH), 7.20 (d, J = 1.8 Hz, 1, ArH), 7.62 (s, 4, ArH), 7.83 (d, J = 16.0 Hz, 1, $HC{=}CCO_2$); infrared (IR) (KBr) 2,955, 1,700, 1,630, 1,430, 1,278, 1,220, 825 cm^{-1}.

4-[(3-Hydroxy-5,6,7,8-tetrahydro-3,5,5,8,8-tetramethyl-2-naphthalenyl)carboxyamido]benzoic acid (SR11281) was synthesized as follows. (i) Fries rearrangement ($AlCl_3$, 130°C [23]) of 2-acetoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene produced 1-(3-hydroxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)ethanone (90%). (ii) Protection of the phenolic group as the benzyl ether (benzyl bromide, K_2CO_3, acetone, reflux; 92%) and oxidation of the acetyl group (NaOCl, EtOH, reflux) gave 3-benzyloxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-carboxylic acid (30%). (iii) The carboxylic acid was converted (oxalyl chloride, CH_2Cl_2) to the acyl chloride and treated (pyridine-benzene) with ethyl 4-aminobenzoate to yield the benzamide (95%). (iv) Ester hydrolysis (NaOH, aqueous EtOH, 25°C; aqueous HCl; 97%) and hydrogenolysis [H_2, Pd(C), EtOH, 25°C; 93%] of the benzyl ether protecting group afforded SR11281: mp, 275 to 278°C; ^1H NMR (300 MHz, $^2HCCl_3$) δ 1.29 (s, 6, CMe_2), 1.32 (s, 6, CMe_3), 1.69 (s, 4, CH_2CH_2), 6.93 (s, 1, ArH), 7.69 (s, 1, ArH), 7.74 (d, 2, J = 8.8 Hz, ArH), 8.08 (d, 2, J = 8.8 Hz, ArH); IR (KBr) 3,330, 1,686, 1,530, 1,419, 1,174 cm^{-1}.

4-[1-(5,6,7,8-Tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl)-2-methylpropenyl]benzoic acid (SR11345) was prepared by Suzuki Pd(0)-catalyzed coupling {$Pd[P(C_6H_5)_3]_4$, $NaHCO_3$, aqueous $MeO(CH_2)_2OMe$, reflux; 58%} (8) between 5,6,7,8-tetrahydro-3,5,5,8,8-pentamethylnaphthalene-2-boronic acid and methyl (1-bromo-2-methylpropenyl)benzoate and hydrolysis (KOH, aqueous EtOH; aqueous HCl; 91%). The first intermediate was synthesized in two steps by bromination (Br_2, $CHCl_3$, 25°C; 80%) of 5,6,7,8-tetrahydro-3,5,5,8,8-pentamethylnaphthalene, followed by conversion to the arylboronic acid [*n*-butyllithium (*n*-BuLi), tetrahydrofuran (THF), −78°C to ambient temperature; $B(OMe)_3$, −78°C to ambient temperature; aqueous NH_4Cl; 86%]. The second intermediate was obtained from methyl (2-methylpropenyl)benzoate by bromination (Br_2, CH_2Cl_2; 100%) and dehydrobromination {1,8-diazabicyclo[5.4.0]undec-7-ene, $MeO(CH_2)_2OMe$, 25°C; 90%} to yield SR11345: mp, 246 to 247°C; ^1H NMR

($^2HCCl_3$) δ 1.25 (s, 12, $4CH_3$), 1.64 (s, 3, CH_3), 1.66 (s, 3, CH_3), 1.88 [s, 4, $(CH_2)_2$], 1.99 (s, 3, CH_3), 7.00 (s, 1, ArH), 7.03 (s, 1, ArH), 7.25 (d, J = 8.3, 2, ArH), 7.98 (d, J = 8.3, 2, ArH); IR (KBr) 3,500 to 2,300 (OH), 1,687 (C = O), 1,606 (C = C) cm^{-1}; chemical ionization high-resolution mass spectrum (CI-HRMS) (NH_3) calculated for $C_{26}H_{32}O_2 + NH_4^+$, 394.2746; found, 394.2751.

4-(1-Amino-5,6,7,8-tetrahydro-5,5,8,8-tetramethylanthracen-2-yl)benzoic acid (SR11350) was synthesized by nitration (HNO_3, acetic anhydride-acetic acid [Ac_2O-HOAc], −10°C) of 6-bromo-1,2,3,4-tetrahydro-1,1,4,4-tetramethylanthracene to give after chromatographic separation (silica, CH_2Cl_2-hexanes) of isomers 6-bromo-1,2,3,4-tetrahydro-1,1,4,4-tetramethyl-5-nitroanthracene (26%), which was coupled {$Pd[P(C_6H_5)_3]_4$, $NaHCO_3$, aqueous dimethyl ether (DME), 80°C; 83%} to 4-carbethoxyphenylboronic acid to afford ethyl 4-(1-nitro-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-anthracen-2-yl)benzoate. Hydrogenation [H_2, Pd(C), EtOAc, 25°C; 95%) to the amine and ester hydrolysis (KOH, aqueous EtOH; 85°C; aqueous HCl; 93%) yielded SR11350 as the HCl: salt mp, 267 to 268°C; ^1H NMR (300 MHz, Me_2SO-$^2H_6$) δ 1.40 (s, 6, CH_3), 1.44 (s, 6, CH_3), 1.77 (s, 4, CH_2), 7.25 (d, J = 8.6 Hz, 1, ArH) 7.55 (d, J = 8.2 Hz, 1, ArH), 7.55 (d, J = 8.2 Hz, 2, ArH), 7.90 (s, 1, ArH), 8.11 (d, J = 8.2 Hz, 2, ArH), 8.21 (s, 1, ArH); electron impact high-resolution mass spectrum (EI-HRMS) calculated for $C_{25}H_{27}NO_2$, 373.2042; found, 373.2040.

6-[3-(1-Adamantyl)-5-methoxyphenyl]naphthalene-2-carboxylic acid (SR11362) was obtained by hydrolysis (KOH, aqueous EtOH, 85°C; aqueous HCl; 90%) of its ethyl ester: mp, 236 to 238°C; ^1H NMR (300 MHz, $^2HCCl3$) δ 1.79 (m, 6, CH_2), 1.98 (m, 6, CH_2), 2.12 (m, 3, CH), 3.87 (s, 3, OCH_3), 6.96 (s, 1, ArH), 7.02 (s, 1, ArH), 7.28 (s, 1, ArH), 7.75 (m, 1, ArH), 7.96 (m, 3, ArH), 8.12 (d, J = Hz, 1, ArH), 8.68 (s, 1, ArH); EI-HRMS calculated for $C_{28}H_{28}O_3$, 412.2038; found, 412.2030.

2-(4-Carboxyphenyl)-6,7,8,9-tetrahydro-6,6,9,9-tetramethylbenzo[g]quinoline-1-oxide (SR11365) was prepared by acetylation (Ac_2O, Et_3N, EtOAc, 25°C; 100%) of 5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-amine to the amide, which on reaction with excess Vilsmeier reagent {$[POC]_3$, 8.5 equivalents; alerts dimethylformamide (DMF) 3.5 equivalents; alerts $(CH_2Cl)_2$, 0°C]; 25 to 85°C; aqueous $NaHCO_3$; 61%} produced 2-chloro-6,7,8,9-tetrahydro-6,6,9,9-tetramethylbenzo[g]quinoline-3-carboxaldehyde, which on coupling {$Pd[P(C_6H_5)_3]_4$, $NaHCO_3$, aqueous DME, 80°C; 71%} with 4-carbethoxyphenylboronic acid yielded ethyl 4-[3-formyl-6,7,8,9-tetrahydro-6,6,9,9-tetramethylbenzo-[g]quinolin-2-yl]benzoate. Oxidation of the formyl group to the carboxylic acid with concomitant base hydrolysis of the ester (Ag_2O, NaOH, aqueous EtOH-THF, 25°C; H_3O^+; 100%) gave the dicarboxylic acid, which on thermal decarboxylation (315°C; 93%) yielded 4-[6,7,8,9-tetrahydro-6,6,9,9-tetramethylbenzo-(g)quinolin-2-yl]benzoic acid, which has also been synthesized by another route (26). Esterification ($SOCl_2$, reflux; MeOH-C_5H_5N-$C_6H_5CH_3$; aqueous $NaHCO_3$; 91%), *N*-oxidation (3-$ClC_6H_4CO_3H$, $CHCl_3$, 25°C; 85%), and ester hydrolysis (NaOH, aqueous EtOH, 70°C; aqueous citric acid; 72%) yielded SR11365: mp, >300°C; ^1H NMR (300 MHz, Me_2SO-$^2H_6$) δ 1.42 (s, 12, CH_3), 1.80 (s, 4, CH_2), 7.68 (d, J = 9 Hz, 1, ArH), 7.95 (d, J = 9 Hz, 1, ArH), 8.10 (d, J = 8 Hz, 2, ArH), 8.14 (s, 1, ArH), 8.15 (d, J = 8 Hz, 2H, ArH), 8.63 (s, 1, ArH); EI-HRMS calculated for $C_{24}H_{25}NO_3$, 375.1834; found, 375.1832.

Cell culture. Breast cancer cell lines ZR-75-1, T-47D, and MDA-MB-231 were obtained from the American Type Culture Collection. ZR-75-1 and T-47D cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, and MDA-MB-231 and CV-1 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Growth inhibition assay. To study anchorage-dependent growth inhibition, cells were seeded at 500 cells per well in 96-well plates and treated with solvent control (dimethyl sulfoxide-EtOH) or with 10^{-6} M retinoids (or 10^{-7} M *trans*-RA) in solvent. Media were changed every 48 h. After treatment for 10 days, the number of viable cells were determined by their capacity to convert the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) into a blue formazan product, using a cell proliferation-cytotoxicity assay kit (Promega, Madison, Wis.) (46).

RNA preparation and Northern blot. For Northern blot analysis, total RNAs were prepared by the guanidine hydrochloride-ultracentrifugation method (34). About 30-μg aliquots of total RNAs from different cell lines treated with or without 10^{-6} M retinoids (or 10^{-7} M *trans*-RA) were fractionated on 1% agarose gel, transferred to nylon filters, and probed with the ^{32}P-labeled ligand-binding domain of receptor cDNAs as previously described (34). To determine that equal amounts of RNA were used, the filters were also probed with β-actin.

Plasmids, receptor proteins, and nuclear extract preparation. The nur77 expression vector was constructed by cloning the nur77 cDNA fragment into pECE or pBluescript vector as described previously (66). The construction of the chloramphenicol acetyltransferase (CAT) reporter containing the RARβ promoter (*Bgl*II-*Bam*HI fragment) and expression vectors for RARα, RARβ, and RXRα have been described elsewhere (21, 67, 68). The RXRα N-terminal deletion mutant (ΔRXR10) was constructed by deleting 61 amino acid residues from its N-terminal end as described previously (71). Receptor proteins were synthesized by an in vitro transcription-translation system using rabbit reticulocyte lysate (Promega) as described previously (67). The relative amount of the translated proteins was determined by [^{35}S]methionine-labeled protein on sodium dodecyl sulfate-polyacrylamide gels by quantitating and then normalizing the amount of incorporated radioactivity relative to the content of methionine in each protein. Nuclear extracts were prepared as previously described (34).

TABLE 1. Retinoid transcriptional activation activity

Retinoid	Relative receptor transactivation (%)[a]			
	RARα	RARβ	RARγ	RXRα
trans-RA	100	100	100	53
9-*cis*-RA				100
SR11277	5	149	65	0
SR11278	13	145	67	0
SR11281	59	123	133	0
SR11365	95	56	48	6
SR11383	66	71	16	7
SR11235	3	27	0	51
SR11246	6	12	9	98
SR11237	1	44	0	95
SR11345	0	−6	−12	107

[a] Transcriptional activation in CV-1 cells, using the $(TREpal)_2$-*tk*-CAT reporter, compared to 1 μM *trans*-RA for RARs or 1 μM 9-*cis*-RA for RXRα as 100%.

Gel retardation assay. The gel retardation assay using in vitro-synthesized proteins or nuclear extracts has been described previously (67, 68). When antibodies were used, 1 μl of anti-nur77 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) or 1 μl of anti-RXR (32) was incubated with receptor protein at room temperature for 30 min prior to the assay.

Transient transfection and CAT assay. To measure transcriptional activation of the RARβ promoter by retinoids, this promoter (*Bgl*II-*Bam*HI fragment) was linked to the CAT gene reporter (21). The reporter plasmid and β-galactosidase expression vector (pCH110; Pharmacia) (100 ng each) with or without the RARβ expression vector were transiently transfected into CV-1 cells by the calcium phosphate precipitation method (34). Cells were grown in the presence or absence of 10^{-6} M retinoids or 10^{-7} M *trans*-RA. Transfection efficiency was normalized to β-galactosidase activity. The data shown are the means of three separate experiments.

Stable transfection. RARα cDNA was cloned into the pRc/CMV expression vector (Invitrogen, San Diego, Calif.) as described elsewhere (34). The resulting recombinant constructs were then stably transfected into MDA-MB-231 breast cancer cells by the calcium phosphate precipitation method and screened by using G418 (Gibco BRL, Grand Island, N.Y.). The levels of exogenous RARα expression were determined by Northern blotting.

Apoptosis analysis. Cells were treated with or without 10^{-6} M retinoids (or 10^{-7} M *trans*-RA). After 48 h, cells were trypsinized, washed with phosphate-buffered saline (PBS; pH 7.4), and fixed in 1% formaldehyde in PBS. After washing with PBS, cells were resuspended in 70% ice-cold EtOH and immediately stored at −20°C overnight. Cells were then labeled with biotin-16-dUTP by terminal deoxynucleotidyltransferase (TdT) and stained with avidin-fluorescein isothiocyanate (Boehringer, Mannheim, Germany). Fluorescently labeled cells were analyzed using a FACScater-Plus as described previously (34). Representative histograms are shown.

RESULTS

RXR-selective retinoids inhibit the growth and induce the apoptosis of *trans*-RA-resistant MDA-MB-231 but not *trans*-RA-sensitive ZR-75-1 cells. Because *trans*-RA effectively inhibited the growth and induced the apoptosis of *trans*-RA-sensitive, estrogen-dependent ZR-75-1 breast cancer cells, whereas it had little effect on *trans*-RA-resistant, estrogen-independent MDA-MB-231 breast cancer cells (34), RAR- and RXR-class selective retinoids (Table 1) were evaluated for the ability to inhibit the growth and induce the apoptosis of these cell lines. At 10^{-6} M, SR11278, SR11281, SR11277, SR11383, and SR11365 activated only the RARs, not RXRα, on the $(TREpal)_2$-*tk*-CAT reporter construct (67, 68), as determined by transient transfection in CV-1 cells. SR11237, SR11246, and SR11235 activated both RXRα and RARβ, whereas SR11345 activated only RXRα (Table 1). Both breast cancer cell lines were treated for 10 days with 10^{-6} M the indicated class-selective retinoid alone or the combination of RXR-selective SR11345 and a RAR-selective retinoid. Cell viability was determined by the MTT assay. As shown in Fig. 1, the RAR-selective retinoids strongly inhibited ZR-75-1 cell growth (55

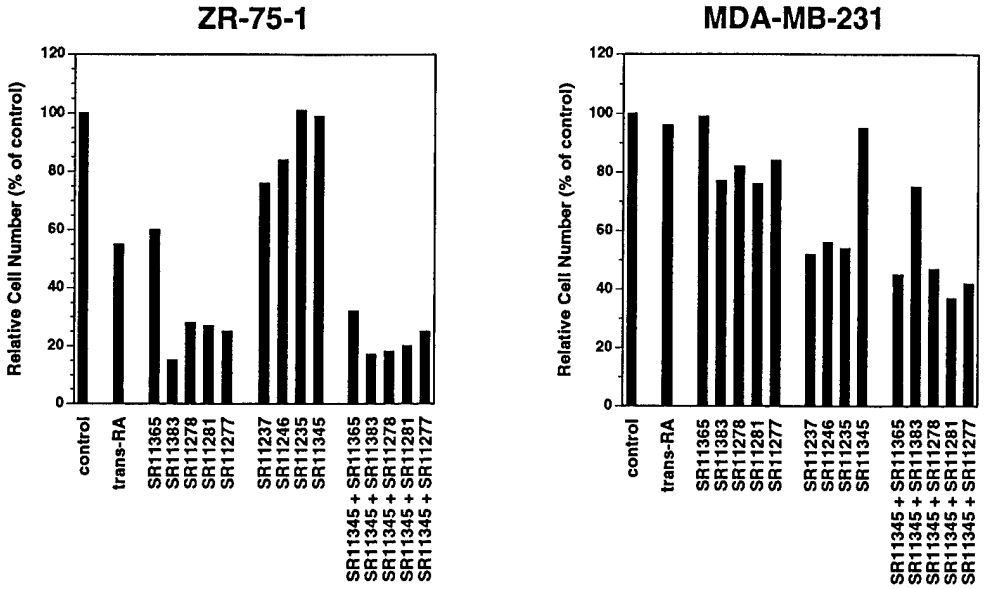


FIG. 1. Growth-inhibitory effects of retinoids on *trans*-RA-sensitive, estrogen-dependent ZR-75-1 and *trans*-RA-resistant, estrogen-independent MDA-MB-231 breast cancer cells. Cells (800 cells/well) were seeded in 96-well plates and treated with the indicated retinoids (10^{-6} M) or *trans*-RA (10^{-7} M) alone or in combination for 10 days. The number of viable cells was determined by the MTT assay.

to 75%). In contrast, the RXR-selective retinoids were far less effective inhibitors (8 to 25%) of growth. ZR-75-1 cell growth inhibition by any of the RAR-selective retinoids was only slightly enhanced by the RXR-selective SR11345. Therefore, growth inhibition of *trans*-RA-sensitive ZR-75-1 cells by retinoids is mediated mainly by the RAR pathway, not the RXR pathway, a finding which is consistent with previous observations (34, 55). Interestingly, in MDA-MB-231 cells, RXR-selective SR11237, SR11246, or SR11235 at 10^{-6} M inhibited growth (45 to 50%) more effectively than any RAR-selective retinoid, which was a poor inhibitor (<20%) (Fig. 1). The more RXR-specific retinoid SR11345 was less effective, with only 8% inhibition. However, when it was used together with one of the RAR-selective retinoids, growth inhibition was increased to 40 to 55%. These results indicate that activation of both RAR and RXR signaling pathways is required for effective cancer cell growth inhibition.

We next investigated the apoptosis-inducing effects of RAR-selective SR11365 and *trans*-RA and of RXR-selective SR11246 in ZR-75-1 and MDA-MB-231 cells by the TdT assay (Fig. 2). *trans*-RA at 10^{-7} M and SR11365 at 10^{-6} M significantly induced apoptosis of ZR-75-1 cells, producing 58 and 27% apoptosis, respectively, whereas RXR-selective SR11246 produced only about 5% apoptotic cells (Fig. 2A). About 44% of MDA-MB-231 cells underwent apoptosis on treatment with SR11246, but apoptosis was not significant on treatment with *trans*-RA (4%) or SR11365 (6%) (Fig. 2B). Together, these results demonstrated that RXR-selective retinoids can induce growth inhibition and apoptosis of *trans*-RA-resistant MDA-MB-231 cells, whereas RAR-selective retinoids are more effective in *trans*-RA-sensitive cells.

Induction of RARβ in *trans*-RA-resistant breast cancer cells by RXR-selective retinoids. We previously demonstrated that RARβ expression levels in breast cancer cells correlated with the extent of growth inhibition and apoptosis induction by *trans*-RA (34). To determine whether growth inhibition and apoptosis induction by RXR-selective retinoids in *trans*-RA-resistant MDA-MB-231 cells were also associated with their induction of RARβ, we compared the effect of RXR-selective retinoids SR11246 and SR11345 and RAR-selective SR11365, as well as the SR11345 and SR11365 combination, on RARβ expression in these cells (Fig. 3). For comparison, *trans*-RA-sensitive ZR-75-1 and T-47D cells were studied. Both *trans*-RA and RAR-selective SR11365, but not RXR-selective SR11246 or SR11345, induced RARβ expression in ZR-75-1 and T-47D cells. However, RXR-selective SR11246 and SR11345 induced RARβ expression in *trans*-RA-resistant MDA-MB-231 cells at a level comparable to that observed with *trans*-RA or SR11365. A further induction of RARβ was observed when MDA-MB-231 cells were treated with both RXR-selective SR11345 and RAR-selective SR11365. These results demonstrate that activation of RXR by RXR-selective retinoids induced RARβ in *trans*-RA-resistant MDA-MB-231 cells, while these retinoids were unable to activate the RXR-pathway for inducing RARβ in *trans*-RA-sensitive ZR-75-1 and T-47D breast cancer cells. Thus, induction of RARβ by RXR-selective retinoids may contribute to the effects of these retinoids on growth inhibition and apoptosis induction in *trans*-RA-resistant MDA-MB-231 cells.

RXR-selective retinoids activate the RARβ promoter through RXR-nur77 heterodimers. RXR ligands can regulate gene expression through RXR homodimers (68) or certain RXR heterodimers, such as RXR-RAR (4, 31), RXR-LXR (63), or RXR-nur77 (15, 49). Regulation of gene expression by RXR-nur77 heterodimers occurs through their binding to DR-5 type RAREs (15, 49). Because the βRARE in the RARβ promoter is a DR-5 type RARE and contains an NBRE (15, 49, 64), we investigated whether induction of RARβ expression in MDA-MB-231 cells (Fig. 3) could be due to apparent activation of RXR-nur77 heterodimers on the βRARE by RXR-selective retinoids. nur77 alone did not show any clear binding to the βRARE under our experimental conditions but in the presence of RXR produced a strong complex, whose binding was largely affected by either anti-RXR or anti-nur77 antibody (Fig. 4A). For a better distinction between RXR-RAR and RXR-nur77 heterodimers, an RXRα mutant with a deletion of

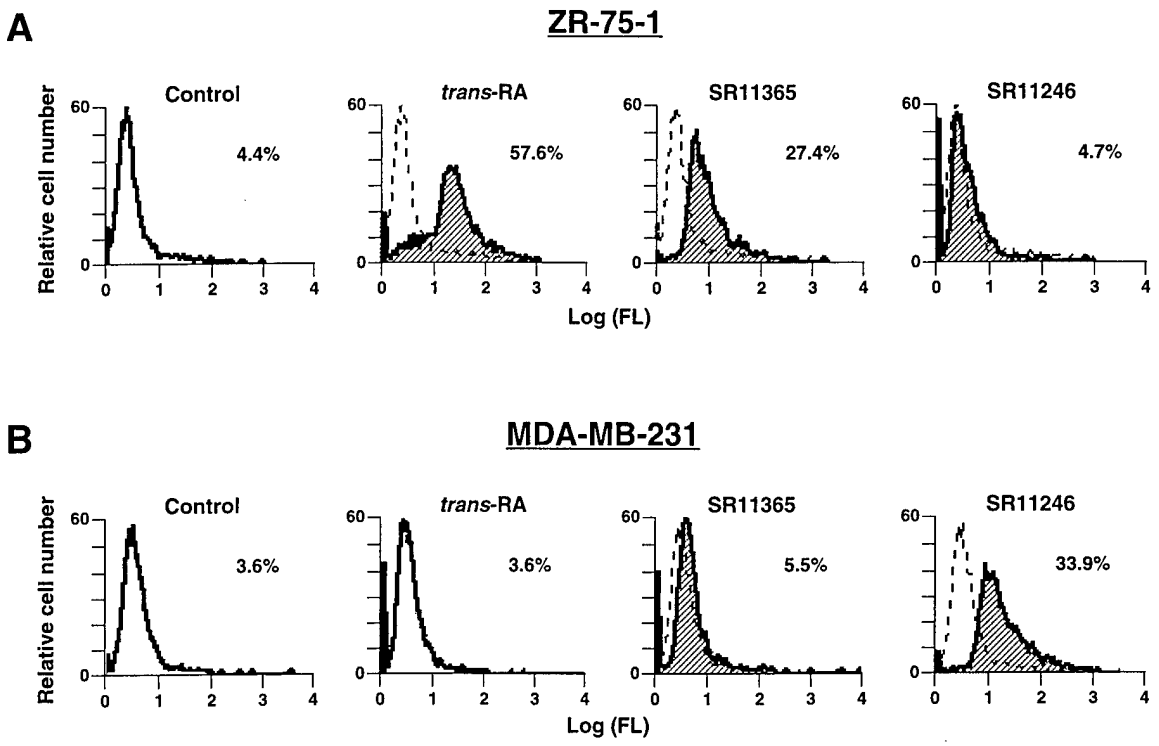


FIG. 2. Induction of apoptosis by retinoids in *trans*-RA-sensitive, estrogen-dependent ZR-75-1 (A) and *trans*-RA-resistant, estrogen-independent MDA-MB-231 (B) breast cancer cells. Breast cancer cells were grown in the presence of the indicated retinoids at 10^{-6} M or *trans*-RA at 10^{-7} M for 48 h. DNA fragmentation was determined by the TdT assay. Representative histograms show relative apoptotic cell number. FL, fluorescence.

68 amino acid residues from its N-terminal end (71) was used. The deletion did not affect heterodimerization properties of the RXR with RAR or nur77 (data not shown). The binding of the RXR-nur77 heterodimers to the βRARE was comparable to that of the RXR-RAR heterodimers (Fig. 4A). Thus, the unique structure of the βRARE permits binding of both RXR-RAR and RXR-nur77 heterodimers, as previously observed (15, 49). We next carried out transient transfection assays in CV-1 cells, using the RARβ promoter linked to the CAT gene (21) as a reporter. As shown in Fig. 4B, cotransfection of the RXRα expression vector did not induce reporter transcriptional activity in response to RXR-selective SR11246 or SR11345, a result that suggests that RXR homodimers do not activate the RARβ promoter as previously observed (68). Cotransfection of the nur77 expression vector clearly induced reporter activity in response to these RXR-selective retinoids but not to RAR-selective *trans*-RA or SR11365. When nur77 and RXR expression vectors were cotransfected, a stronger induction of reporter activity was observed when cells were treated with the RXR-selective retinoids but not with *trans*-RA or SR11365. To determine the effect of RARα on RXR-nur77 activity, we cotransfected the RARα expression vector together with RXRα and nur77. Addition of RARα strongly inhibited RXR-selective retinoid-induced reporter activity but significantly enhanced *trans*-RA and SR11365 activity (Fig. 4B). The inhibition of SR11246 and SR11345 activity by RARα is likely due to competition of RARα and nur77 for heterodimerization with RXR and binding to the βRARE, which suggests that RXR-selective retinoids SR11246 and SR11345 cannot activate RARβ promoter through RXR-RAR heterodimers in CV-1 cells.

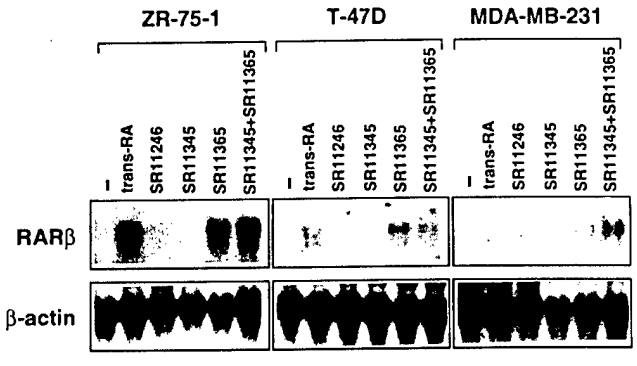
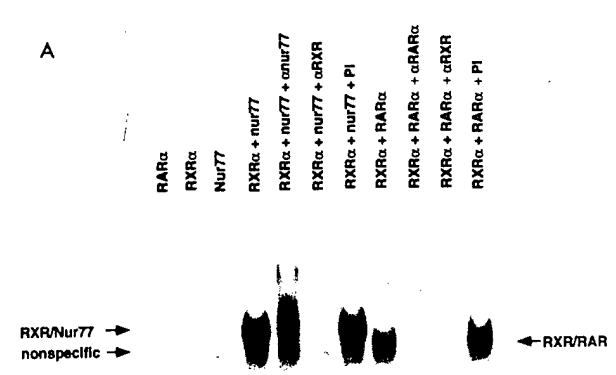


FIG. 3. Effects of RAR-selective SR11365 and RXR-selective SR11246 and SR11345 on RARβ gene expression in *trans*-RA-sensitive, estrogen-dependent ZR-75-1 and T-47D and *trans*-RA-resistant, estrogen-independent MDA-MB-231 cells. RNAs were prepared from cells treated with 10^{-6} M RXR-selective SR11246 or SR11345, RAR-selective SR11365, or a combination of SR11345 and SR11365 for 24 h and analyzed for RARβ expression by Northern blotting. For comparison, the expression of the β-actin is shown.

Competitive binding of RXR-RAR and RXR-nur77 heterodimers to the βRARE. Our observation that RARα inhibited the transactivation activities of RXR-selective SR11246 and SR11345 on the βRARE (Fig. 4B) suggests that RARα may compete with nur77 for heterodimerization with RXR and thus prevent nur77 from binding to the βRARE. We therefore carried out gel retardation assay using the βRARE as a probe (Fig. 5). When RARα protein was added, binding of RXRα-nur77 heterodimers to the βRARE decreased in a RARα dose-dependent manner. Excess amounts of RARα permitted binding of RXRα-RARα heterodimers. Similarly, increasing nur77 protein levels inhibited RXR-RAR heterodimer binding



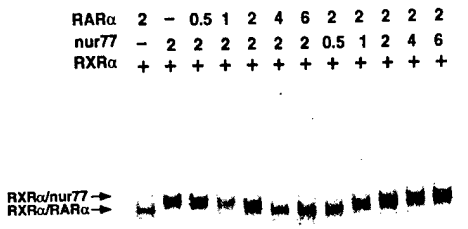


FIG. 5. Competition binding of RXR-RAR and RXR-nur77 heterodimers to the βRARE. To analyze the effect of RARα on RXR-nur77 heterodimer binding to the βRARE, in vitro-synthesized N-terminally deleted RXRα protein (1 μl) was incubated with in vitro-synthesized nur77 (2 μl) in the absence or presence of the indicated amounts (microliters) of in vitro-synthesized RARα protein and analyzed by gel retardation using the βRARE probe. To analyze the effect of nur77 on RXRα/RARα heterodimer binding, in vitro-synthesized RXRα protein (1 μl) was incubated with in vitro-synthesized RARα (2 μl) in the absence or presence of the indicated amounts (μl) of in vitro-synthesized nur77 and analyzed by gel retardation.

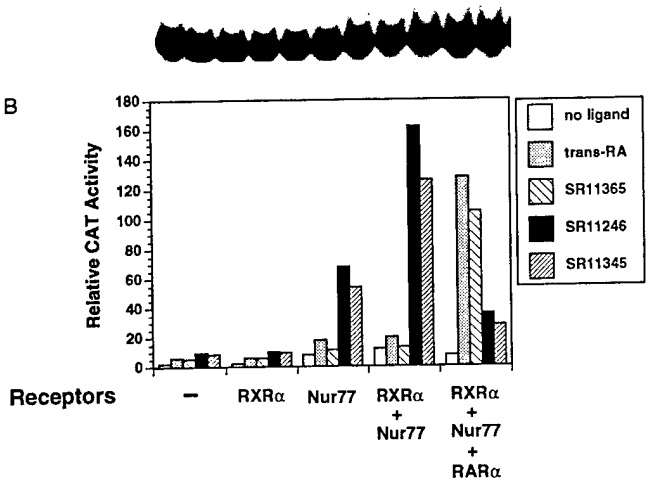


FIG. 4. Binding and transactivation of nur77-RXRα and RARα-RXRα on the βRARE. (A) Binding of the nur77-RXRα and RXRα-RARα heterodimers to the βRARE. Equal amounts of in vitro-synthesized nur77 and an N-terminally deleted RXRα (see Materials and Methods) alone or combined were incubated with the βRARE probe at room temperature for 10 min. Mixtures were analyzed by gel retardation. Anti-RXR (αRXR), anti-RARα (αRARα), or anti-nur77 (αnur77) was incubated with receptor proteins for 30 min at room temperature before performance of the assay. For a control, receptor proteins were also incubated with preimmune serum (PI). The βRARE probe sequence was GTAGGGTTCACCGAAAGTTCAGTC (the NBRE is in boldface). (B) nur-77-promoted transactivation of RARβ in CV-1 cells. A CAT reporter containing the RARβ promoter (19) was transiently transfected into CV-1 cells with the receptor expression vector RXRα (20 ng), nur77 (100 ng), or RARα (200 ng). After 24 h, cells were treated with the indicated retinoids (10^{-6} M) or *trans*-RA (10^{-7} M) for 24 h, and CAT activities were determined as described elsewhere (65).

to the βRARE but enhanced RXR-nur77 heterodimer binding. Thus, RARα and nur77 compete for dimerization with RXR and binding to the βRARE. These data suggest that the relative levels of RARα and nur77 regulate binding of RXR-nur77 and RXR-RAR to the βRARE. We therefore determined whether different levels of RARα, RXRα, and nur77 were expressed in *trans*-RA-sensitive ZR-75-1 and in *trans*-RA-resistant MDA-MB-231 cells. Consistent with previous observations (34, 51, 54, 55, 60), RARα levels were much higher in ZR-75-1 cells than in MDA-MB-231 cells (Fig. 6). However, RXRα and nur77 were equally expressed in both cell lines independently of the presence of *trans*-RA. The high RARα levels in ZR-75-1 cells suggest that binding of RXRα-RARα heterodimers to the βRARE may preferentially occur to mediate the effects of RAR-selective ligands, while the low RARα levels in MDA-MB-231 cells suggest that RXR-nur77 heterodimer may be predominantly formed with the βRARE to mediate the inhibitory effects of RXR-selective ligands.

Stable expression of RARα in *trans*-RA-resistant MDA-MB-231 cells favors the RAR pathway over the RXR pathway. Low levels of RARα in MDA-MB-231 cells should enhance RXR-nur77 heterodimer formation to mediate the effects of RXR-selective retinoids. To determine whether overexpression of RARα would allow RXR-RAR heterodimer formation but inhibit that of RXR-nur77, we stably transfected RARα into

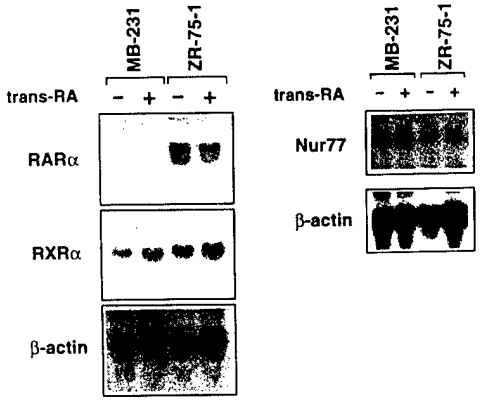


FIG. 6. Expression of RARα, RXRα, and nur77 in *trans*-RA-sensitive ZR-75-1 and *trans*-RA-resistant MDA-MB-231 cells. Total RNAs, prepared from cells treated with 10^{-7} M *trans*-RA for 24 h, were analyzed for the expression of RARα, RXRα, and nur77 by Northern blotting. For comparison, the expression of the β-actin is shown.

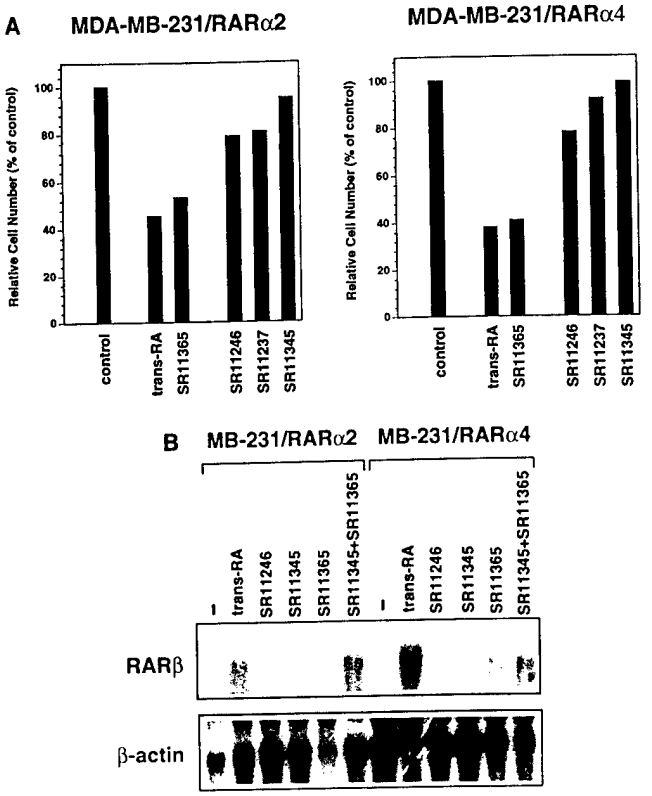


FIG. 7. Effect of stable expression of RARα on growth inhibition and RARβ induction by RXR-selective and RAR-selective retinoids in *trans*-RA-resistant MDA-MB-231 cells. (A) RARα modulates sensitivity of MDA-MB-231 cells to RAR-selective and RXR-selective retinoids. Stable clones, expressing introduced RARα, were seeded at 800 cells/well in 96-well plates and treated with the indicated retinoid (10^{-6} M) or *trans*-RA (10^{-7} M) for 10 days. The number of viable cells was determined by the MTT assay. (B) Stable expression of RARα regulates RARβ expression in response to RAR-selective and RXR-selective retinoids in MDA-MB-231 cells. Stable MDA-MB-231 clones expressing high levels of RARα (MB-231/RARα2 and MB-231/RARα4) were treated with the indicated retinoid (10^{-6} M) alone or in combination and analyzed for the expression of RARβ by Northern blotting. The expression of β-actin was used as the control.

this cell line. Two stable clones (MDA-MB-231–RARα2 and MDA-MB-231–RARα4) that expressed high levels of transfected RARα (data not shown) were analyzed for their responses to RAR and RXR class-selective retinoids (Fig. 7A). Compared to their effects in the parental MDA-MB-231 cells (Fig. 1), RAR-selective *trans*-RA at 10^{-7} M and SR11365 at 10^{-6} M were far more potent inhibitors of the stable clones, showing 46 to 62% inhibition, while RXR-selective SR11246, SR11237, and SR11345 were less effective inhibitors, with less than 21% inhibition. We also investigated the effect of RARα on RARβ expression in MDA-MB-231 cells by Northern blotting (Fig. 7B). In contrast to their effects on the parental cells (Fig. 3), RAR-selective *trans*-RA and SR11365 strongly induced RARβ expression in both clones, while RXR-selective retinoids SR11246 and SR11345 did not. These results demonstrate that low RARα expression in MDA-MB-231 cells is responsible for the increased ability of RXR-selective retinoids and the decreased ability of RAR-selective retinoids to induce RARβ expression and growth inhibition. The fact that the extent of growth inhibition and RARβ expression level by these receptor class-selective retinoids in the stable clones were similar to those in *trans*-RA-sensitive ZR-75-1 and T-47D cells (Fig. 1 and 3) suggests that the differential effects of retinoids on certain *trans*-RA-sensitive and -resistant breast cancer cell lines depend on different levels of RARα expression.

Binding of nuclear proteins from ZR-75-1 and MDA-MB-231 cells to the βRARE. To provide direct evidence that relative levels of RXR, RARα, and nur77 in *trans*-RA-sensitive ZR-75-1 and *trans*-RA-resistant MDA-MB-231 cells allowed different complex formation on the βRARE, we prepared nuclear proteins from ZR-75-1 and MDA-MB-231 cells and analyzed their binding to the βRARE (Fig. 8). Nuclear proteins

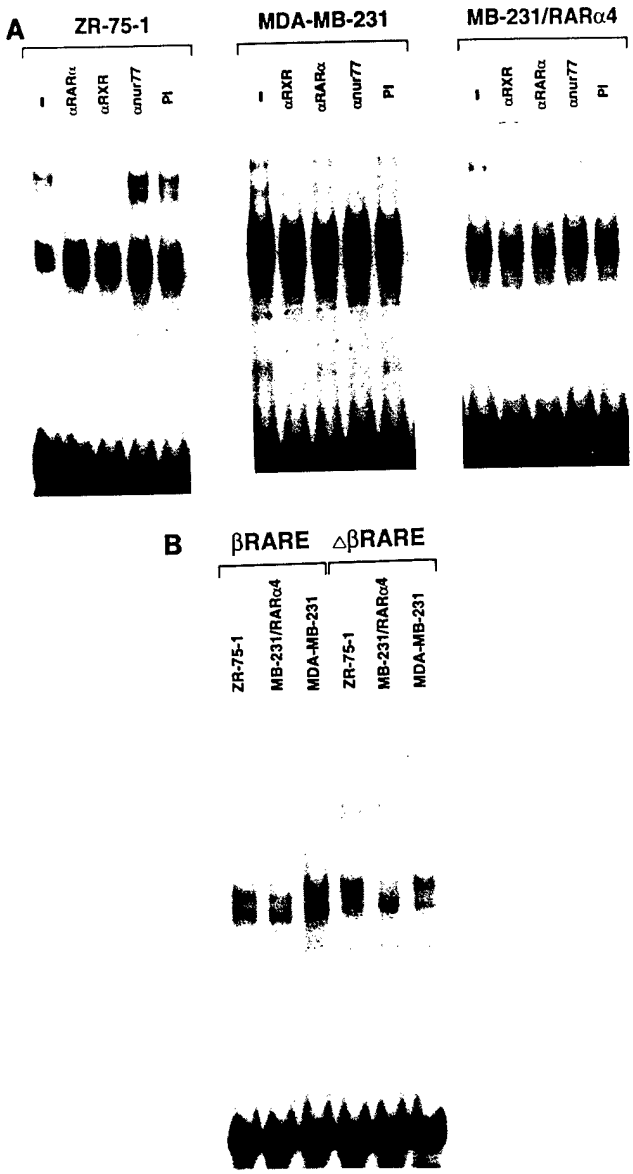


FIG. 8. βRARE binding activities of nuclear proteins from ZR-75-1, MDA-MB-231, and MDA-MB-231–RARα4 cells. (A) Nuclear proteins from ZR-75-1 (2 μg), MDA-MB-231 (5 μg), and MDA-MB-231–RARα4 (2 μg) cells were analyzed by gel retardation assay using βRARE as a probe. When antibody (designated as in Fig. 4A) was used, it (1 μl) was incubated with nuclear proteins for 30 min at room temperature before performance of the assay. Preimmune serum (PI) was used for control. (B) Comparison of βRARE and ΔβRARE binding of nuclear proteins from ZR-75-1 (2 μg), MDA-MB-231 (2 μg), and MDA-MB-231/RARα4 (2 μg) cells by gel retardation assay. The ΔβRARE probe sequence was GTAGGGTTCACCGTGAGTTCAGTC (mutated nucleotides compared to βRARE are indicated in boldface).

from ZR-75-1 cells formed several strong complexes with the βRARE. When they were incubated with anti-RXR antibody, the slowly migrating complexes were inhibited. When anti-RARα antibody was used, some of the slowly migrating complexes were also abolished. However, anti-nur77 antibody did not show any detectable effect on the binding. These data demonstrate that RXR and RARα are mainly responsible for βRARE binding in ZR-75-1 cells. When nuclear proteins from MDA-MB-231 cells were analyzed, we observed weak βRARE binding complexes, which could be inhibited by anti-RXR and anti-nur77 antibodies but not by anti-RARα antibody. Thus, expression of RXR and nur77 in MDA-MB-231 cells (Fig. 6) could contribute to the βRARE binding activities. To determine whether overexpression of RARα in MDA-MB-231 cells could prevent RXR-nur77 binding as we observed by using in vitro-synthesized receptor proteins (Fig. 5), we analyzed the binding of nuclear proteins prepared from MDA-MB-231–RARα4 cells. As shown in Fig. 8A, the nuclear proteins formed a strong complex with the βRARE, which could be completely inhibited by either anti-RXR antibody or anti-RARα antibody but not by anti-nur77 antibody, indicating that overexpression of RARα in MDA-MB-231 cells inhibited RXR-nur77 heterodimer binding and permitted RXR-RAR heterodimer binding. To further determine the nature of the binding complexes from different cell lines, we used a mutated βRARE (ΔβRARE), in which two adenine nucleotides in the spacing region of the βRARE were mutated. The mutations do not affect binding of RXR-RAR heterodimers but abolish RXR-nur77 binding (66) and thereby allow distinction of RXR-RAR heterodimers from RXR-nur77 heterodimers. When nuclear proteins from ZR-75-1, MDA-MB-231, and MDA-MB-231–RARα4 were analyzed on the ΔβRARE, we observed a strong binding of nuclear proteins from ZR-75-1 and MDA-MB-231–RARα4 cells, similar to that observed with the βRARE. In contrast, nuclear proteins from MDA-MB-231 cells did not show any detectable binding on the ΔβRARE, demonstrating that the binding complex that we observed on the βRARE might represent RXR-nur77 heterodimer binding.

DISCUSSION

Breast cancer cell growth inhibition and apoptosis induction by RXR-selective retinoids. Although conventional retinoids show promise in animal models as preventive agents against breast cancer, their anticancer effects appear to be limited to *trans*-RA-sensitive tumors, whereas the more aggressive, estrogen-independent tumors are usually refractory (14, 34, 54, 55, 60). In this study, we demonstrated that several RXR-selective retinoids inhibit the growth and induce the apoptosis of *trans*-RA-resistant MDA-MB-231 cells (Fig. 1), provided that they are also capable of activating the RARs or are used in combination with RAR-selective retinoids. Efficient growth inhibition by RXR-selective retinoids appears to be cell type specific because it was observed in *trans*-RA-resistant MDA-MB-231 cells but not in *trans*-RA-sensitive ZR-75-1 cells (Fig. 1). In ZR-75-1 cells, RAR-selective SR11365 was much more effective than RXR-selective SR11246 in inhibiting the growth and inducing apoptosis (Fig. 1 and 2). Thus, different retinoid signaling pathways preferentially operate in *trans*-RA-sensitive and *trans*-RA-resistant breast cancer cells to mediate retinoid-induced growth inhibition.

Although the RXR pathway is clearly involved in *trans*-RA-resistant MDA-MB-231 breast cancer cell growth inhibition, a clear growth inhibition by RXR-selective retinoids required a longer period of treatment (Fig. 1) than that by RAR-selective retinoids, which usually inhibit the growth of *trans*-RA-sensitive breast cancer cells over a period of 3 to 4 days of treatment (data not shown). This observation suggests that the effects of RXR-selective retinoids may involve a mechanism different from that utilized by RAR-selective retinoids. We showed that activation of RXR alone was insufficient for growth inhibition and that activation of RAR appeared to be required, as indicated by our observation that RXR-selective SR11237, SR11246, and SR11247, which at 1 μM have the ability to slightly activate the RARβ (Table 1), can significantly inhibit the growth of MDA-MB-231 cells (Fig. 1), whereas the far more RXR-selective SR11345, which activates RXRα comparably to the other RXR-selective retinoids (Table 1), did not significantly inhibit MDA-MB-231 cell growth (Fig. 1). Furthermore, RXR-selective SR11345 and RAR-selective SR11365, which alone were ineffective inhibitors, on combination strongly prevented MDA-MB-231 cell growth (Fig. 1). The biological activities of RXR-selective retinoids have been described in several studies (6, 17, 47). Activation of RXR was reported as essential for inducing apoptosis in HL-60 leukemia cells (47). RXR-selective retinoid LGD1069 effectively inhibited the tumor development in the *N*-nitroso-*N*-methylurea-induced rat mammary tumor model (17). Because of their increased efficacy against malignant, *trans*-RA-resistant, estrogen-independent breast cancer growth, RXR-selective retinoids may be useful for chemoprevention and chemotherapy of breast cancer.

How RXR-selective retinoids inhibit the growth and induce the apoptosis of *trans*-RA-resistant MDA-MB-231 and other cancer cells remains to be fully elucidated. Induction of growth inhibition and apoptosis of breast cancer cells by retinoids may involve different retinoid receptors and different mechanisms, depending on types of retinoids and cell lines (11, 12, 33, 34, 53–55, 60). The effects of *trans*-RA may be mediated by RARα and RARβ (33, 34, 53–55, 60), whereas activation of RARγ may be required for other retinoids, such as 4-HPR (11, 12). Our data presented here suggest that induction of RARβ may be involved. This is consistent with previous observations that RARβ could mediate the growth-inhibitory effect of *trans*-RA in breast cancer cells (33, 34, 53). RARβ was induced by *trans*-RA only in *trans*-RA-sensitive ZR-75-1 and T-47D, not in *trans*-RA-resistant MDA-MB-231, breast cancer cells (34). In addition, introduction of RARβ into RARβ-negative MDA-MB-231 breast cancer cell lines restored *trans*-RA-induced growth inhibition (33, 34, 53), while inhibition of RARβ activity in the RARβ-positive ZR-75-1 cells with an antisense construct abolished growth inhibition by *trans*-RA (34). Furthermore, enhancement of RARβ levels has been found to correlate with senescence in normal mammary epithelial cells (58). In *trans*-RA-sensitive ZR-75-1 and T-47D cells, RARβ expression was strongly induced by RAR-selective *trans*-RA and SR11365, which also inhibited growth and induced apoptosis, whereas RARβ expression was not induced by RXR-selective SR11246 and SR11345, which only poorly inhibited growth and only weakly induced apoptosis (Fig. 3). The fact that RXR-selective retinoids could induce RARβ expression in *trans*-RA-resistant MDA-MB-231 cells (Fig. 3) suggests that induction of RARβ may contribute to their effects on MDA-MB-231 cells. However, because *trans*-RA and SR11365 induced RARβ to levels similar to those induced by RXR-selective retinoids SR11246 and SR11345 in MDA-MB-231 cells (Fig. 3) but were poor growth inhibitors (Fig. 1), mechanisms other than RARβ induction may also be involved.

SR11345 synergized with RAR-selective retinoids to inhibit MDA-MB-231 cell growth (Fig. 1). Such synergism of RAR- and RXR-selective retinoids has recently been observed in the

activation of several RA-responsive genes, including RARβ, during embryonal carcinoma cell differentiation (52) and in NB4 acute promyelocytic leukemia cells (4). The synergism that we observed here may in part arise from induction and activation of RARβ. Growth inhibition of MDA-MB-231 cells may require both induction and activation of RARβ because RXR-specific SR11345 is a much less effective inhibitor than other RXR-selective retinoids (Fig. 1), which also slightly activate RARβ (Table 1). Enhanced induction of RARβ in MDA-MB-231 cells by the combination of RXR-specific SR11345 and RAR-selective SR11365 (Fig. 3) may also contribute to their synergistic growth inhibition.

Regulation of RARβ expression. The βRARE in the RARβ promoter is responsible for regulating RARβ expression by retinoids (10, 21, 57). Our observation in gel shift assays that efficient binding to the βRARE occurred by heterodimerization of RXRα with nur77 or RARα but not by either receptor alone (Fig. 4A) confirms that the βRARE binds both RXR-RAR and RXR-nur77 heterodimers (15, 49). Activation of the βRARE by *trans*-RA is caused by binding and activation of RAR-RXR heterodimers, in which RXR functions as a silent partner (15, 30, 38), while activation of the βRARE by RXR-selective retinoids occurs on binding of RXR-nur77 heterodimers (15, 49). Cotransfection of RXRα and nur77 strongly activated RARβ promoter activity in response to RXR-selective SR11246 or SR11345 but not to RAR-selective *trans*-RA or SR11365 (Fig. 4B). Thus, the βRARE can be activated by either a RAR-selective or RXR-selective retinoid signaling pathway through binding of RXR-RAR or RXR-nur77, respectively. This is reminiscent of a previous observation that the βRARE bound strongly to an RXR-containing complex other than RXR-RAR in S91 melanoma cell extracts (56). Because nur77 expression is induced by several growth factors having different signal transduction pathways (20, 39), the binding and activation of the βRARE by nur77 and retinoid receptors will mediate the convergence of retinoid and growth factor signaling pathways.

Activation of the βRARE by the RAR pathway or RXR pathway depends on the breast cancer cell type. In *trans*-RA-sensitive cell lines such as ZR-75-1 and T-47D, the expression of RARβ was highly induced by RAR-selective retinoids but not by RXR-selective retinoids, whereas in *trans*-RA-resistant MDA-MB-231 cells, RARβ expression was induced by RXR-selective retinoids (Fig. 3). Because both RXRα and nur77 are well expressed in MDA-MB-231 cells (Fig. 6), induction of RARβ by RXR-selective retinoids is likely mediated by activation of RXR-nur77 heterodimers on the βRARE. This is further supported by our observation that binding of nuclear proteins prepared from MDA-MB-231 cells to the βRARE may represent RXR-nur77 heterodimers (Fig. 8). Such cell-type-specific activation of the βRARE has been observed previously. In CV-1 cells, RXR-selective retinoids did not appreciably transactivate the DR-5 βRARE even in the presence of transfected RAR and RXR (15, 30). However, in P19 or F9 embryonal carcinoma cells, RXR ligands contributed to transcriptional activation of genes containing DR-5 elements (4, 40, 52). The cell-type-specific activation of the βRARE is likely due to the relative levels of nuclear receptors that bind the βRARE and modulate its activity.

Although RXR-selective retinoids could induce RARβ expression in MDA-MB-231 cells, we observed a stronger induction of RARβ when cells were treated with a combination of RAR-selective and RXR-selective retinoids (Fig. 3). The strong induction of RARβ by the combination may be due to the additive effect of RXR-RAR and RXR-nur77 heterodimers, since RAR-selective retinoids by themselves could also slightly induce RARβ probably due to low levels of RARα expressed in these cells. Recently, it was reported that binding of RAR-selective retinoids to RXR-RAR heterodimers allowed binding and activation of RXR-RAR heterodimers by RXR-selective retinoids (4, 40). Thus, it is also possible that the strong induction of RARβ that we observed by the combination of RAR-selective and RXR-selective retinoids is due to activation of RXR-RAR heterodimers prebound with RAR-selective retinoid.

RARα regulates both RAR and RXR pathways. *trans*-RA-sensitive and -resistant breast cancer cell lines display different responses to retinoid receptor class-selective ligands. RAR-selective retinoids are potent RARβ inducers and growth inhibitors in *trans*-RA-sensitive ZR-75-1 cells, while RXR-selective retinoids effectively induce RARβ and inhibit the growth of *trans*-RA-resistant MDA-MB-231 cells (Fig. 1 and 3). The observation that the βRARE can be activated by either RXR-RAR or RXR-nur77 suggests that the pathway that mediates growth inhibition and RARβ induction may largely depend on the relative levels of RARα, RXRα, and nur77. In lung cancer cell lines, nur77 expression is associated with *trans*-RA resistance (66) and could be critical in regulating RAR and RXR activities. However, in breast cancer cell lines, ZR-75-1 and MDA-MB-231 cell lines express similar levels of RXRα and nur77, while RARα varies, being highly expressed in the former and underexpressed in the latter (Fig. 6), as has been previously observed (34, 51, 55, 60). Thus, RARα levels appear to be most important for determining whether the RAR or RXR pathway will regulate growth inhibition by retinoids. High RARα levels in *trans*-RA-sensitive ZR-75-1 cells may permit formation of RXR-RAR heterodimers that bind to the βRARE (Fig. 5 and 8) to mediate the effects of RAR-selective retinoids in inducing RARβ expression and growth inhibition (34) but prevent RXRα from forming RXR-nur77 heterodimers (Fig. 5 and 8) so that RXR-selective retinoids are unable to inhibit growth or induce apoptosis despite the abundant expression of nur77. In contrast, low RARα levels in *trans*-RA-insensitive MDA-MB-231 cells (Fig. 6) allow formation of RXR-nur77 heterodimers (Fig. 8) that bind to the βRARE to mediate RARβ expression and may be responsible for growth inhibition in the presence of RXR-selective retinoids. The importance of RARα levels in determining the regulatory pathway is supported by our gel retardation (Fig. 5 and 8) and transfection assay results (Fig. 4B). Gel retardation indicates that binding of RXR-RAR or RXR-nur77 heterodimers to the βRARE largely depends on RARα protein levels (Fig. 5 and 8). Overexpression of RARα in MDA-MB-231 cells allowed binding of RXR-RARα heterodimers and prevented binding of RXR-nur77 heterodimers to the βRARE (Fig. 5 and 8). In transient transfection assays, cotransfection of RARα inhibited RXR-selective retinoid-induced RXR-nur77 heterodimer activity on the RARβ promoter (Fig. 4B). Furthermore, stable expression of RARα in MDA-MB-231 cells strongly enhanced growth inhibition (Fig. 7A) and RARβ induction (Fig. 7B) by RAR-selective retinoids and decreased the inhibitory effects of RXR-selective retinoids. Thus, high RARα levels favor formation of RAR-RXR heterodimers and the RAR signaling pathway in breast cancer cells, while low RARα levels favor the formation of nur77-RXR heterodimers and the RXR signaling pathway. This retinoid signaling switch may play an important role in regulating breast cancer cell growth in response to different growth factor and retinoid stimuli.

In summary, we have demonstrated that RXR-selective retinoids inhibit the growth and induce the apoptosis of *trans*-RA-resistant MDA-MB-231 breast cancer cells, which appears to

be mediated through RXR-nur77 heterodimers that bind and activate the βRARE in the presence of RXR-selective retinoids, resulting in induction of RARβ, which may then be activated by RAR-selective retinoids to initiate secondary biological responses. RXR-nur77 heterodimer formation in *trans*-RA-resistant MDA-MB-231 cells is favored by very low RARα levels, whereas high expression of RARα in *trans*-RA-sensitive ZR-75-1 cells favors formation of RXR-RAR heterodimers that bind and activate the βRARE in response to RAR-selective ligands. Thus, the convergence and switch of RAR-dependent and RXR-dependent signaling on the βRARE is very likely regulated by relative RARα levels. Our findings that an RXR signaling pathway can mediate growth inhibition and apoptosis induction and the additive to synergistic effects of a RAR-selective and RXR-selective retinoid combination on *trans*-RA-resistant MDA-MB-231 cell growth may provide a therapeutic opportunity to inhibit the growth of more invasive, *trans*-RA-resistant breast cancer by using lower retinoid doses to reduce toxicity.

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Interaction of BAG-1 with Retinoic Acid Receptor and Its Inhibition of Retinoic Acid-induced Apoptosis in Cancer Cells*

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BAG-1 (also known as RAP46) is an anti-apoptotic protein, which has been shown previously to interact with a number of nuclear hormone receptors, including receptors for glucocorticoid, estrogen, and thyroid hormone. We show here that BAG-1 also interacts with retinoic acid receptor (RAR). Gel retardation assays demonstrated that *in vitro* translated BAG-1 protein could effectively inhibit the binding of RAR but not retinoid X receptor (RXR) to a number of retinoic acid (RA) response elements (RAREs). A glutathione *S*-transferase-BAG-1 fusion protein also specifically bound RAR but not RXR. Interaction of BAG-1 and RAR could also be demonstrated by yeast two-hybrid assays. In transient transfection assays, co-transfection of BAG-1 expression plasmid inhibited the transactivation activity of RAR/RXR heterodimers but not RXR/RXR homodimers. When stably expressed in breast cancer cell lines, BAG-1 inhibited binding of RAR/RXR heterodimer to a number of RAREs and suppressed RA-induced growth inhibition and apoptosis. In addition, RA-induced suppression of Bcl-2 expression was abrogated by overexpression of BAG-1. These results demonstrate that BAG-1 can regulate retinoid activities through its interaction with RAR and suggest that elevated levels of BAG-1 protein could potentially contribute to retinoid resistance in cancer cells.

Development of a multicellular organism requires tightly regulated cellular processes, such as proliferation, differentiation, and cell death. Failure to maintain the balance among these fundamental and mechanistically related processes may result in abnormal cell growth, as seen in cancer cells where cell death is often inhibited (1, 2). Retinoids, a group of natural and synthetic vitamin A derivatives, are currently used to treat epithelial cancer and promyelocytic leukemia and are being evaluated for prevention and therapy of other human cancers (3, 4). The anti-cancer effects of retinoids are mainly due to their inhibition of cell proliferation, induction of cell differentiation, and promotion of apoptosis. Retinoids alone or in combination with other stimuli induce apoptosis during normal development and in different types of cancer cells *in vitro* (5–11). However, it remains largely unknown how retinoid-induced apoptosis is regulated.

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the retinoic acid receptors (RARs)[1] and retinoid X receptors (RXRs). RARs and RXRs are encoded by three distinct genes (α, β, and γ) and are members of the steroid/thyroid/retinoid hormone receptor superfamily that function as ligand-activated transcription factors (12–14). 9-*cis* RA is a high affinity ligand for both RARs and RXRs, whereas all-*trans*-RA (*trans*-RA) is a ligand for only RARs. RARs and RXRs primarily function as RXR/RAR heterodimers that bind to a variety of RA response elements (RAREs) and regulate their transactivation activities.

Regulation of gene expression either positively or negatively by nuclear hormone receptors is modulated by additional factors. Some of them appear to provide a direct link to the core transcriptional machinery and to modulate chromatin structure (15), such as SRC-1 (16), SUG-1 (17), TIF-1 (18), RIP-140 (19), N-CoR (25), SMRT (26), TIF-2 (20), GRIP-1 (21), p160 (22), CBP (23), AIB1 (8), and ACTR (24), whereas a number of other cellular proteins, such as AP-1, have been implicated in the regulation of nuclear hormone receptor activity, probably through their interaction with receptors (27).

The involvement of retinoid receptors in retinoid-induced apoptosis has been demonstrated in several studies. Expression of RARβ may be involved in the apoptosis of mesenchyme of the interdigital regions during mouse limb development (28). RARβ is required for RA-induced apoptosis of breast cancer (5) and lung cancer (7) cells, whereas activation of RXR is essential for RA-induced HL-60 cell apoptosis (29). In 4-HPR-induced apoptosis, activation of RARγ may be involved (30, 31), whereas regulation of activation-induced apoptosis of T-cells by 9-*cis* RA requires activation of both RARs and RXRs (32).

Although much interest has been directed to the role of retinoid-induced apoptosis in both physiological and pathological processes, very little is known regarding regulation of the process. It is believed that apoptosis, once triggered, proceeds through a central death pathway in which specific cellular proteases and endonucleases are activated (1, 2, 33). Members of the Bcl-2 family play an important role in the regulation of the central death pathway. Bcl-2 can suppress induction of apoptosis in many systems, whereas Bax promotes apoptosis. In addition, several other proteins that modulate Bcl-2 activity by interacting with Bcl-2 have been described (1, 2, 33). One of these genes, *BAG-1* (for Bcl-2-associated anti-death gene 1), was cloned from a murine embryo cDNA library using a pro-

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[1] The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid response elements; TR, thyroid hormone receptor; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; GST, glutathione *S*-transferase; tk, thymidine kinase; MHC, myosin heavy chain; CAT, chloramphenicol acetyltransferase.

tein-protein interaction technique (34). Two differently localized BAG-1 isoforms, the long BAG-1 isoform and the short BAG-1 isoform, generated by alternative translation initiation are expressed in mammalian cells (35). Whether two isoforms act differently remains to be determined. Recent studies demonstrated that co-expression of BAG-1 and Bcl-2 in Jurkat lymphoid cells, NIH 3T3 fibroblasts, and melanoma cells promoted the survival of these cells in response to a variety of apoptotic stimuli (34, 36, 37). In addition to Bcl-2, BAG-1 also interacts with Raf-1 (38), resulting in activation of its kinase activity. Furthermore, BAG-1 can interact with hepatocyte growth factor receptor and platelet-derived growth factor receptor and enhance the ability of these receptors to transduce signals for cell survival (39). These observations suggest that BAG-1 may function as an adaptor to mediate the interaction between survival factors and apoptotic machinery and may also play a role in regulating cellular proliferation. The recent observation that BAG-1 binds tightly to Hsp70/Hsc70-family proteins and modulates their chaperone activity (40–42) suggests that the ability of BAG-1 to alter the activities of diverse groups of proteins involved in cell growth control may be attributed to its effects on Hsp70/Hsc70 proteins.

Interestingly, the human BAG-1 homolog (also known as RAP46) was cloned from a human liver cDNA library by virtue of its interaction with the glucocorticoid receptor (43). *In vitro*, RAP46 interacts with a number of nuclear hormone receptors, including estrogen receptor and thyroid hormone receptor (TR) (43). Since molecular chaperones are known to play an important role in controlling the activity of many members of the steroid/thyroid/retinoid receptor family (44), it is possible that BAG-1 could alter the function of these transcriptional regulators. Before this report, however, it was unknown whether BAG-1 regulates the activities of the nuclear hormone receptors and whether BAG-1 interacts with retinoid receptors.

Here we demonstrate that short BAG-1 isoform interacts with the RAR but not the RXR both *in vitro* and *in vivo*. GST pull-down and the yeast two-hybrid assays show that BAG-1 directly interacts with RAR but not RXR. Moreover, BAG-1 inhibits RAR/RXR heterodimer DNA binding and suppresses RA-induced transactivation activity of RARs on various RAREs. Overexpression of BAG-1 in MCF-7 and ZR-75-1 breast cancer cells reduces the ability of *trans*-RA to inhibit the growth and induce apoptosis, as well as its modulation of Bcl-2 expression. Taken together, our results demonstrate that BAG-1 can physically interact with RARs and is an important component in the retinoid response pathway. Our findings suggest that this protein-protein interaction may play a role in the regulation of retinoid-induced growth inhibition and apoptotic processes, potentially contributing to retinoid resistance in cancer.

MATERIALS AND METHODS

Cell Culture—Monkey kidney CV-1 cells and breast cancer MCF-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, and ZR-75-1 breast cancer cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum.

Growth Inhibition Assay—Cells were seeded at 1,000–2,000 cells/well in 96-well plates and treated 24 h later with various concentrations of *trans*-RA for 7 days. Media and *trans*-RA were changed every 48 h. Relative viable cell number was determined using the MTT assay (52).

Apoptosis Analysis—For the terminal deoxynucleotidyl transferase assay (5), cells were treated with or without 10^{-6} M *trans*-RA. After 48 h, cells were trypsinized, washed with phosphate-buffered saline, fixed in 1% formaldehyde in phosphate-buffered saline, washed with phosphate-buffered saline, resuspended in 70% ice-cold ethanol, and stored at −20 °C overnight. Cells were then labeled with biotin-16-dUTP by terminal transferase and stained with avidin-fluorescein isothiocyanate (Boehringer Mannheim). Fluorescently labeled cells were analyzed using a FACScater-Plus. Representative histograms are shown.

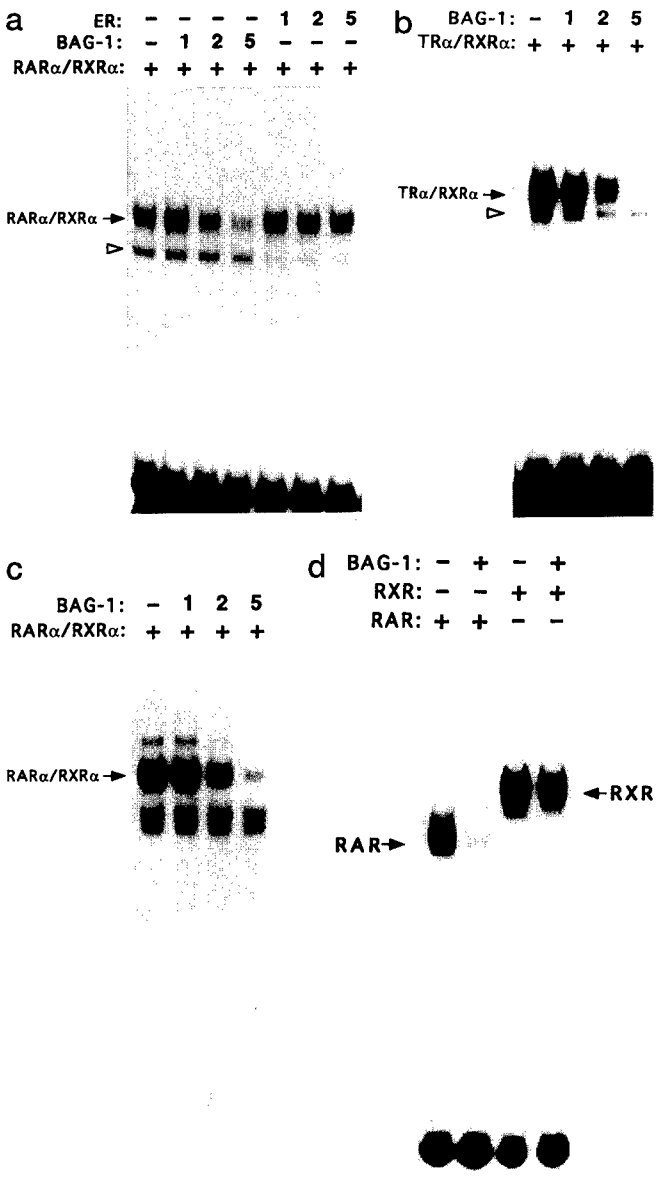

FIG. 1. **Inhibition of RAR DNA binding by BAG-1.** *a*, inhibition of RAR/RXR heterodimer binding by BAG-1. *In vitro* synthesized RARα and RXRα were preincubated with the indicated molar excess of *in vitro* synthesized BAG-1 or estrogen receptor (*ER*). Unprogrammed reticulocyte lysate was used to maintain an equal protein concentration in each reaction. After this preincubation, the reaction mixtures were incubated with ^{32}P-labeled TREpal and analyzed by the gel retardation assay. The *open arrow* indicates nonspecific binding. *b*, inhibition of TRα/RXRα binding by BAG-1. *In vitro* synthesized TRα and RXRα were preincubated with the indicated molar excess amount of *in vitro* synthesized BAG-1 and analyzed by the gel retardation assay using the TREpal as a probe. *c*, inhibition of RARα/RXRα binding on the βRARE by BAG-1. *In vitro* synthesized RARα and RXRα were preincubated with the indicated molar excess amount of *in vitro* synthesized BAG-1 and analyzed by the gel retardation assay using the β RARE as a probe. *d*, inhibition of DNA binding of bacterially expressed RAR but not RXR by BAG-1. Bacterially expressed RARγ or RXRα protein was preincubated with 6 μl of *in vitro* synthesized BAG-1 and analyzed by the gel retardation assay using the TREpal as a probe.

Antibodies and Immunoblotting—Cells were lysed in 150 mM NaCl, 10 mM Tris, pH7.4, 5 mM EDTA, 1% Triton X-100 and protease inhibitors phenylmethylsulfonyl fluoride, aprotinin, leupeptins, and pepstatin. Equal amounts of lysates (50 μg) were boiled in SDS sample buffer, resolved by SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membrane. After transfer, the membranes were blocked in TBST (50 mM Tris, pH7.5, 150 mM NaCl, 0.1% Tween 20) containing rabbit anti-Bcl-2 serum. The membranes were then washed

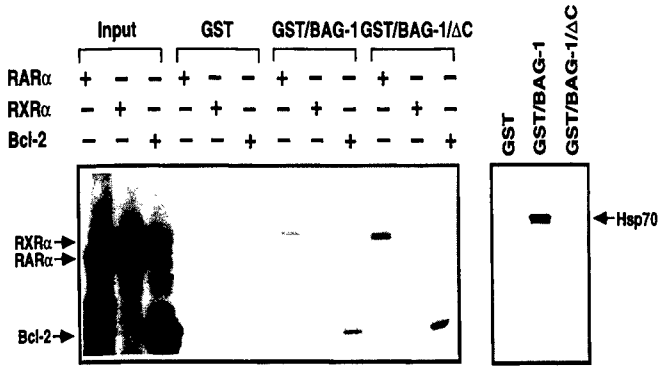

FIG. 2. **Analysis of RAR-BAG-1 interaction by the GST pull-down assay.** BAG-1 or a BAG-1 C-terminal deletion mutant (BAG-1/Δc) was expressed in bacteria using the pGex.4T expression vector. The GST-BAG-1 proteins were immobilized on glutathione-Sepharose beads. As a control, the same amount of GST was also immobilized. ^{35}S-Labeled RARα, RXRα, or Bcl-2 was then mixed with the beads. After extensive washing, the bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The input proteins are shown for comparison (*left panel*). For comparison, binding of GST-BAG-1 and GST-BAG-1/Δc to Hsc70 was shown in *right panel*.

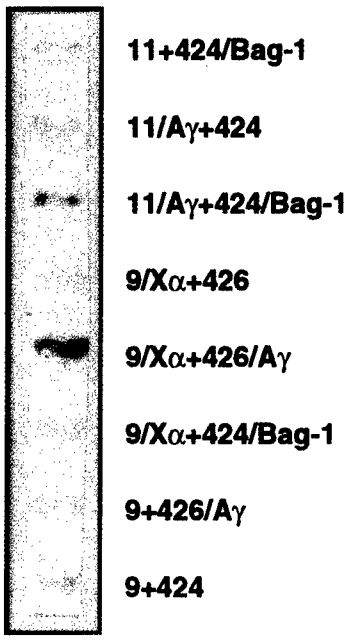

FIG. 3. **RAR and BAG-1 interact in yeast.** The RARα and BAG-1 cDNAs were cloned into the yeast expression vectors pGAD and pGBT, respectively. The resulting expression vectors were introduced into Y190 yeast cells. The yeast transformants were streaked on a filter and assayed for β-galactosidase activity. *11+424/Bag-1*, pGBT and pGAD/BAG-1; *11/Aγ+424*, pGBT/RARγ + pGAD; *11/Aγ+424/Bag-1;* pGBT RARγ + pGAD/BAG-1; *9/Xα+426*, pGBT/RXRα + pGAD; *9/Xα+426/Aγ*, pGBT/RXRα + pGAD/RARγ; *9/Xα+424/Bag-1*, pGBT/RXRα + pGAD/BAG-1; *9+426/Aγ*; pGBT + pGAD/RARγ; *9+424*, pGBT + pGAD.

three times with TBST and then incubated for 1 h at room temperature in TBST containing horseradish peroxidase-linked anti-rabbit immunoglobulin. After three washes in TBST, immunoreactive products were detected by chemiluminescence with an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

Transient and Stable Transfection Assay—For CV-1 cells, 1×10^5 cells were plated per well in a 24-well plates 16–24 h before transfection as described previously (45). For ZR-75-1 cells, 5×10^5 cells/well were seeded in 6-well culture plates. A modified calcium phosphate precipitation procedure was used for transient transfection (45). For CV-1 cells, 100 ng of reporter plasmid, 150 ng of β-galactosidase expression vector (pCH 110, Amersham), and various amounts of BAG-1 expression vector that expresses short BAG-1 isoform (35) were mixed with carrier DNA (pBluescript) to 1,000 ng of total DNA/well. Reporter plasmids βRARE-tk-CAT, TREpal-tk-CAT, and TRE_{MHC}-tk-CAT have been previously described (45–48). For stable transfection, the pRc/CMV-BAG-1 plasmid (34) that expresses short BAG-1 isoform was stably transfected into MCF-7 or ZR-75-1 cells using calcium phosphate precipitation method, followed by selection using G418 (Life Technologies, Inc.) as described (5).

Preparation of Receptor, BAG-1, and Nuclear Protein—cDNAs for RARα, RXRα, estrogen receptor, and BAG-1, which expresses short BAG-1 isoform cloned into pBluescript (Stratagene), were transcribed by using T_7 or T_3 RNA polymerase, and the transcripts were translated in the rabbit reticulocyte lysate system (Promega) as described previously (45). The relative amounts of the translated proteins were determined by separating the [^{35}S]methionine-labeled proteins on SDS-polyacrylamide gels, quantitating the amount of incorporated radioactivity and normalizing it relative to the content of methionine residues in each protein. To synthesize receptor fusion protein, RARγ or RXRα cDNAs were cloned in-frame into the bacterial expression vector pGex.2T (Amersham) as described (45). Preparation and purification of GST-BAG-1 and GST-BAG-1(Δ172–218) fusion proteins has been described (41). Preparation of nuclear extract was described previously (6).

Gel Retardation Assay—Analysis of *in vitro* synthesized or bacterially expressed receptor proteins or nuclear proteins by gel retardation was described previously (45). To analyze the effect of BAG-1 protein, *in vitro* synthesized BAG-1 protein was preincubated with receptor protein at room temperature for 10 min before the gel retardation assay. The oligonucleotides used for gel retardation assays were βRARE (TGTAGGGTTCACCGAAAGTTCAGTC) (46); TREpal (TGAGGTCATGACCTGA) (45); DR-5-RARE(TGTAGGGTTCACACTGAGTTCACTCA); and DR-2-RARE(AGGTCAAAAGGTCAG).

GST Pull-down Assay—To analyze the interaction between BAG-1 and RAR, GST-BAG-1 fusion protein was immobilized on glutathione-Sepharose beads as described (52). As a control, GST prepared under the same conditions was also immobilized. The beads were preincubated with bovine serum albumin (1 mg/ml) at room temperature for 5 min. ^{35}S-Labeled *in vitro* synthesized receptor proteins (2 to 5 μl, depending on translation efficiency) Bcl-2 or Hsc70 were then added to the beads. The beads were then continuously rocked for 1 h at 4 °C in a final volume of 200 μl in EBC buffer (140 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM sodium orthovanadate, and 50 mM Tris, pH 8.0). After washing five times with NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% Nonidet P-40), the bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis.

Two-hybrid Assay—For the yeast two-hybrid assay, the yeast two-hybrid system from CLONTECH Inc. (Palo Alto, CA) was used (52). BAG-1 cDNA was cloned into the yeast expression vector pGAD424 to generate an in-frame fusion with the Gal4 activation domain. RARγ or RXRα cDNAs were cloned into pGBT11 or pGBT9, respectively, to produce an in-frame fusion with Gal4 DNA binding domain. RARγ was also cloned into pGAD426 that contains Gal4 activation domain to study the interaction between RARγ and RXRα. The yeast reporter strain Y190 containing a LacZ reporter plasmid with Gal4 binding sites was used for transformation. β-Galactosidase activity was determined following the conditions provided by the manufacturer.

RESULTS

Inhibition of Retinoid Receptor DNA Binding by BAG-1—We investigated whether BAG-1 could interact with retinoid receptors by studying the effect of BAG-1 protein on binding of retinoid receptors to their target DNA sequences. *In vitro* synthesized RAR and RXR formed a strong RAR/RXR heterodimer complex with the TREpal as described previously (45). When increasing amounts of *in vitro* synthesized short BAG-1 isoform protein were incubated with RAR and RXR, the binding of RAR/RXR heterodimers was inhibited in a BAG-1 concentration-dependent manner (Fig. 1*a*). At a 5 M excess of BAG-1 protein relative to RAR/RXR, the binding was almost completely inhibited. The effect of BAG-1 on RAR/RXR binding was specific because similar amounts of estrogen receptor did not show any effect. BAG-1 also effectively inhibited the binding of TR/RXR to the TREpal probe (Fig. 1*b*), consistent with a prior report that BAG-1 can interact with TR (43). To study whether the inhibitory effect of BAG-1 on RAR/RXR heterodimer binding is specific to the TREpal, we used another RA responsive element (βRARE), which is derived from the RARβ promoter (46). As

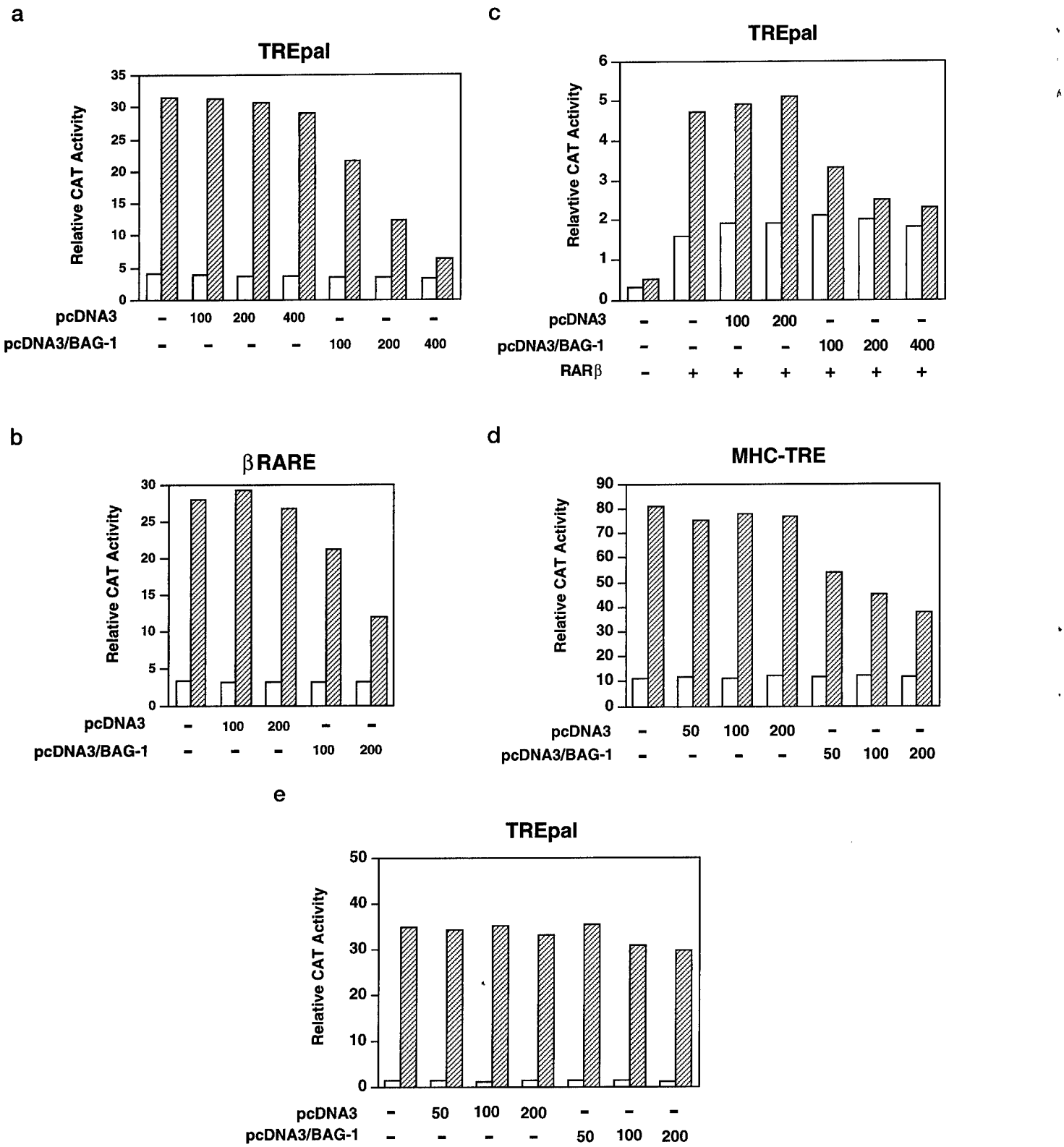

FIG. 4. **Inhibition of transactivation activities of nuclear receptors by BAG-1.** *a*, inhibition of RARα activity on the TREpal by BAG-1. The TREpal-tk-CAT reporter plasmid was co-transfected into CV-1 cells with 100 ng of RARα expression vectors together with the indicated amounts of BAG-1 expression vector or the empty plasmid (pcDNA3) into CV-1 cells. Transfected cells were treated with ▨ or without □ 10^{-7} M *trans*-RA and assayed 24 h later for CAT activity. *b*, inhibition of RARα activity on the βRARE by BAG-1. The βRARE-tk-CAT was co-transfected with 100 ng of RARα expression vectors together with the indicated amounts of BAG-1 expression vector or the empty vector (pcDNA3) into CV-1 cells, Transfected cells were treated with ▨ or without □ 10^{-7} M *trans*-RA and assayed 24 h later for CAT activity. *c*, inhibition of RARβ activity on the TREpal by BAG-1. The TREpal-tk-CAT reporter (47) was co-transfected by either 100 ng of RARβ expression vector together with the indicated amounts of pcDNA3/BAG-1 or pcDNA3. Cells were treated with ▨ or without □ 10^{-7} M *trans*-RA. *d*, inhibition of TR/RXR activities by BAG-1 MHC-TRE-tk-CAT reporter plasmid was co-transfected with 100 ng of TRβ expression vectors together with the indicated amounts of BAG-1 expression vector or the empty vector (pcDNA3) into CV-1 cells. Transfected cells were treated with or without 10^{-7} M T_3 and assayed 24 h later for CAT activity. *e*, effect of BAG-1 on RXR homodimer activity on the TREpal. The TREpal-tk-CAT was cotransfected with 100 ng of RXRα expression vector together with the indicated amount of BAG-1 or empty vector. Cells were treated with ▨ or without □ 10^{-7} M 9-*cis*-RA.

shown in Fig. 1*c*, binding of RAR/RXR on the βRARE was also inhibited by the addition of BAG-1 protein. Similar results were obtained with other RAREs, including CRBPI-RARE and ApoAI-RARE (data not shown). Thus, inhibition of RAR/RXR binding to their target DNA sequences by BAG-1 is independent of RAREs.

We next determined whether inhibition of RAR/RXR heterodimer binding by BAG-1 is due to interaction of BAG-1 with RAR or RXR. Since *in vitro* synthesized RAR or RXR alone does not bind efficiently to RARE, we used bacterially expressed RAR or RXR protein. When a 5-fold molar excess of BAG-1 protein was added, the binding of RARγ was significantly inhibited, whereas binding of RXRα was not affected (Fig. 1*d*). These results suggest that inhibition of RAR/RXR or TR/RXR heterodimer DNA binding by BAG-1 is likely due to its interaction with RAR or TR but not with RXR.

Interaction of BAG-1 with Retinoid Receptors—To further study the interaction between BAG-1 and RAR, we used an *in vitro* GST pull-down assay. A GST-BAG-1 fusion protein was expressed in bacteria and immobilized on glutathione-Sepharose beads. The beads were then incubated with *in vitro* synthesized ^{35}S-labeled RAR or RXR protein. After extensive washing, the mixtures were analyzed on a SDS-polyacrylamide gel. In comparison to the input lane, significant amounts of labeled RAR but not RXR were retained by GST-BAG-1-Sepharose beads but not by GST control beads (Fig. 2). For control, Bcl-2, a known BAG-1 interacting protein (34), bound strongly to GST-BAG-1 beads. We also employed a BAG-1 mutant protein in which the last 47 amino acid residues are deleted from its C-terminal end (41). This mutant protein (BAG-1/Δc) can interact with Bcl-2 but not with Hsc70. Interestingly, labeled RAR but not RXR was also retained by the mutant BAG-1, thus implying that the interaction of BAG-1 with RAR is independent of its binding to Hsc70.

Interaction between RAR and BAG-1 was also evaluated by the two-hybrid assay in yeast. Fig. 3 shows that co-transformation of BAG-1 and RARγ significantly activated the reporter in β-gal filter assay, whereas co-transformation of BAG-1 and RXRα did not. Interaction between BAG-1 and RARγ was specific because co-transformation of either BAG-1 or RARγ with the corresponding empty vector did not activate the reporter gene. Thus, RAR and BAG-1 also interact in intact cells.

Inhibition of Transactivation Activity of Retinoid Receptors by BAG-1—To further examine the BAG-1-RAR interaction, we studied the effects of the short BAG-1 isoform on RAR transactivation activity on a number of RAREs by transient transfection assay. When CV-1 cells were transiently transfected with RARα expression vector together with either TREpal-tk-CAT (Fig. 4*a*) or βRARE-tk-CAT (Fig. 4*b*), *trans*-RA-induced reporter gene activity was markedly inhibited by co-transfection of BAG-1 expression plasmid in a concentration-dependent manner. The effect is specific to BAG-1 because co-transfection of similar amounts of empty expression vector (pcDNA3) did not inhibit *trans*-RA-induced gene expression. BAG-1 also showed inhibitory effect on *trans*-RA-induced RARβ activity on the TREpal. However, the *trans*-RA-independent RARβ activity was not affected. This suggests that ligand-dependent RAR activity is more sensitive to the inhibitory effect of BAG-1. A similar inhibitory effect of BAG-1 was also obtained when RARγ expression vector was used (data not shown). We also studied the effects of BAG-1 on thyroid hormone (T_3)-induced TRα activity, and we observed a significant inhibitory effect of BAG-1 on TRα (Fig. 4*d*), consistent with the ability of BAG-1 to bind the TR protein (Fig. 1*b*; Ref. 43). To determine the effect of BAG-1 on RXR homodimer activity, we co-transfected TREpal-tk-CAT reporter plasmid and RXRα expression vector. However, the 9-*cis*-RA-induced RXRα homodimer activity was not affected by co-transfection of BAG-1 (Fig. 4*e*), consistent with our observation that BAG-1 does not interact with RXRα *in vitro* (Figs. 1*d*, 2, and 3).

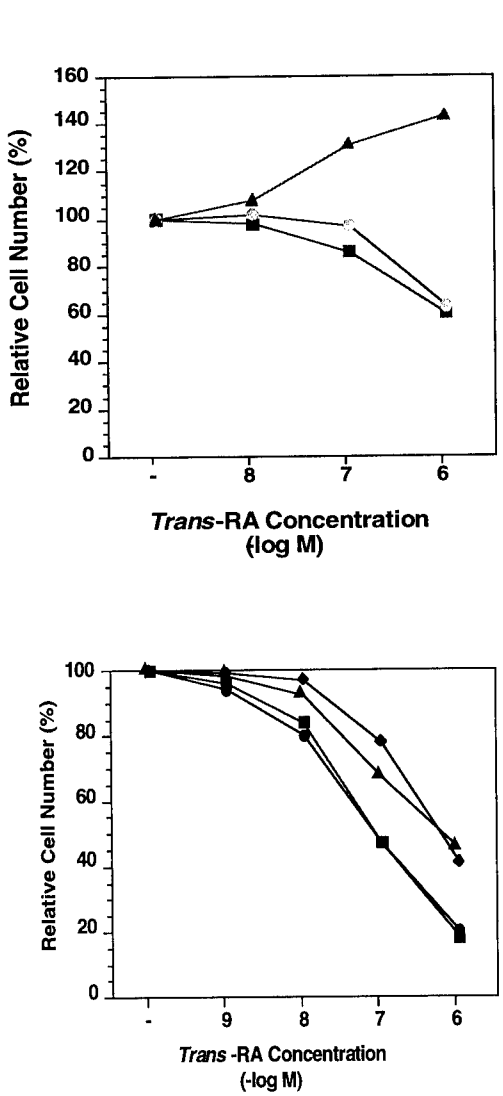

FIG. 5. **Overexpression of BAG-1 reduces *trans*-RA-induced growth inhibition of breast cancer cells.** *a*, effect of constitutive BAG-1 expression on RA-induced growth inhibition in MCF-7 cells. The growth of BAG-1 stable transfectant, MCF-7/BAG-1(#3) (*shaded triangle*), parental MCF-7 cells (*black box*), and MCF-7 cells transfected with empty vector (MCF-7/neo, *shaded circle*) in the absence or presence of the indicated concentration of *trans*-RA was determined by the MTT assay. *b*, effect of constitutive BAG-1 expression on *trans*-RA-induced growth inhibition in ZR-75-1 cells. The growth of BAG-1 overexpressing transfectants 75-1/BAG-1(#5) (▲) and 75-1/BAG-1(#6) (♦), parental ZR75-1 cells (■), and ZR75-1 cells transfected with the empty vector (75-1/neo) (●) in the absence or presence of the indicated concentrations of *trans*-RA was determined by the MTT assay.

Overexpression of BAG-1 Inhibits Trans-RA-induced Cancer Cell Apoptosis—The above data suggest that BAG-1 may function as a modulator of *trans*-RA-induced biological responses. We previously showed that *trans*-RA effectively inhibits the growth and induces apoptosis of some human breast cancer cell lines (5). We, therefore, stably expressed BAG-1 in human breast cancer cell lines MCF-7 and ZR-75-1. MCF-7/BAG-1(#3), 75-1/BAG-1(#5), and 75-1/BAG-1(#6) that stably expressed high levels of the transfected BAG-1 plasmid (data not shown) were chosen to examine the effect of BAG-1 overexpression on RA activities. As shown in Fig. 5*a*, *trans*-RA effectively inhibited the growth of parental MCF-7 and ZR-75-1 cells. However,

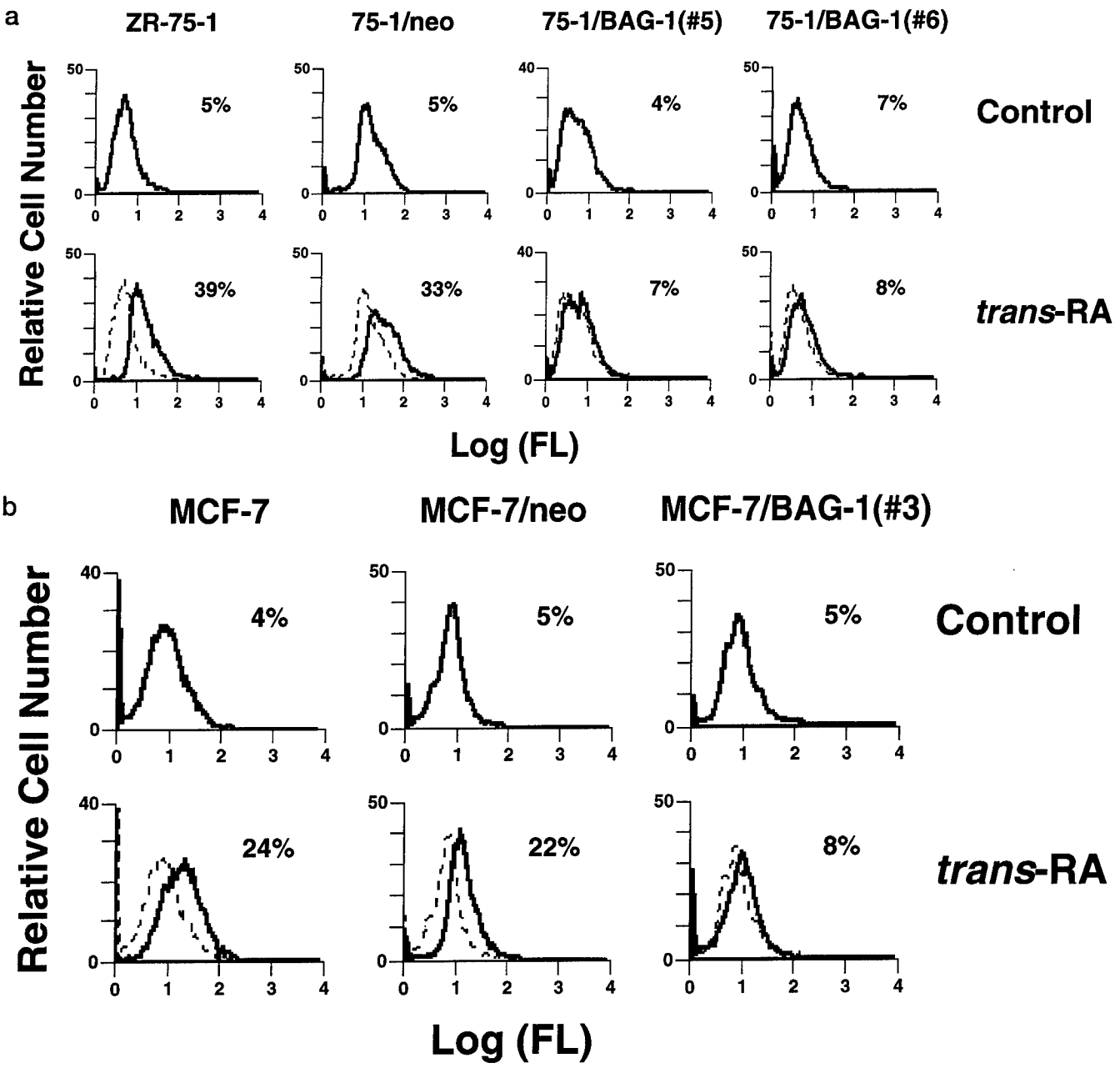

FIG. 6. **Overexpression of BAG-1 inhibits *trans*-RA-induced apoptosis of ZR-75-1 cells.** *a*, inhibition of *trans*-RA-induced apoptosis in ZR-75-1 cells. *b*, inhibition of *trans*-RA-induced apoptosis in MCF-7 cells. Cells were treated with 10^{-6} M *trans*-RA for 48 h, and DNA fragmentation was determined by the terminal deoxynucleotidyl transferase assay. Representative histograms show relative apoptotic cell numbers. *FL*, fluorescence.

the BAG-1-overexpressing clones displayed resistance to the growth inhibitory effects of *trans*-RA. The growth of the MCF-7/BAG-1(#3) was even stimulated by *trans*-RA (Fig. 5*a*). Although *trans*-RA did not stimulate the growth of 75-1/BAG-1(#5) and 75-1/BAG-1(#6) cells, its inhibitory effect on the growth of these cells was significantly reduced as compared with its effect on ZR-75-1 cells (Fig. 5*b*). The effect on *trans*-RA activity observed above was specific because clones stably transfected with the empty vector, MCF-7/neo and 75-1/neo, exhibited similar responses to *trans*-RA as that observed with the parental cell lines. Thus, BAG-1 partially abrogates the growth inhibitory effects of *trans*-RA on human breast cancer cells.

We next investigated the effects of BAG-1 on *trans*-RA-induced apoptosis of ZR-75-1 and MCF-7 cells using the terminal deoxynucleotidyl transferase assay. Extensive DNA fragmentation was induced by *trans*-RA in ZR-75-1 and ZR-75-1/neo cells. In the typical experiment shown in Fig. 6*a*, about 39 and 33% of the ZR-75-1 and ZR-75-1/neo cells underwent apoptosis in response to *trans*-RA, respectively. However, ZR-75-1/BAG-1(#5) and ZR-75-1/BAG-1(#6) cells experienced much less DNA fragmentation under the same conditions, with only about 7 and 8% apoptotic cells, respectively. Similarly, the apoptogenic effect of *trans*-RA on MCF-7 cells was significantly reduced by BAG-1 overexpression (Fig. 6*b*). Thus, overexpression of BAG-1 inhibits *trans*-RA-induced apoptosis in breast cancer cells.

Overexpression of BAG-1 Abrogates Down-regulation of Bcl-2 by Trans-RA—Retinoids have been shown to down-regulate the expression of Bcl-2 in leukemia (11). We therefore studied whether BAG-1 affected RA-regulated expression of Bcl-2 in MCF-7 cells. Bcl-2 was highly expressed in MCF-7 cells, and its expression level was significantly reduced by *trans*-RA as de-

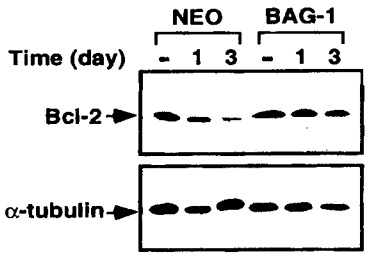

FIG. 7. **Overexpression of BAG-1 prevents inhibition of Bcl-2 expression by *trans*-RA in MCF-7 cells.** Cell lysates prepared from MCF-7/neo (*NEO*) and MCF-7/BAG-1(#3) (*BAG-1*) cells treated with 10^{-6} M *trans*-RA for the indicated time were electrophoresed in a SDS-polyacrylamide gel. After transfer to nitrocellulose membrane, Bcl-2 was detected with rabbit anti-Bcl-2 serum by Western blotting.

termined by immunoblotting. In MCF-7/BAG-1(#3)-stable transfectant, however, treatment of *trans*-RA had little or no effect on Bcl-2 expression (Fig. 7). These data suggest that BAG-1 may inhibit *trans*-RA-induced apoptosis at least in part through its effects on *trans*-RA-regulated genes, such as *Bcl-2*.

To further study the effect of BAG-1 on RAR-mediated gene regulation, we prepared nuclear extract from MCF-7, MCF-7/neo, and MCF-7/BAG-1(#3) cells and analyzed their binding to various RAREs, including βRARE, DR-5-type RARE, and DR-2-type RARE. As shown in Fig. 8*a*, extracts from MCF-7 and MCF-7/neo showed strong binding complex to these RAREs, whereas binding of the slow-migrating complex observed in MCF-7 and MCF-7/neo cells was strongly inhibited in MCF-7/BAG-1(#3) cells. To determine the nature of the slow-migrating complex, extract from MCF-7 cells was incubated with either anti-RAR (α-RAR) or anti-RXR (α-RXR) antibody or nonspecific preimmune serum prior to DNA binding reaction. Fig. 8*b* shows that either anti-RAR or anti-RXR antibody, but not nonspecific serum, completely inhibited the formation of the complex, suggesting that the complex contains RAR and RXR. These data further demonstrate that overexpression of BAG-1 inhibits RAR/RXR binding and suggest that the alteration of RAR transcriptional activity may contribute to the effect of BAG-1 on RA responses.

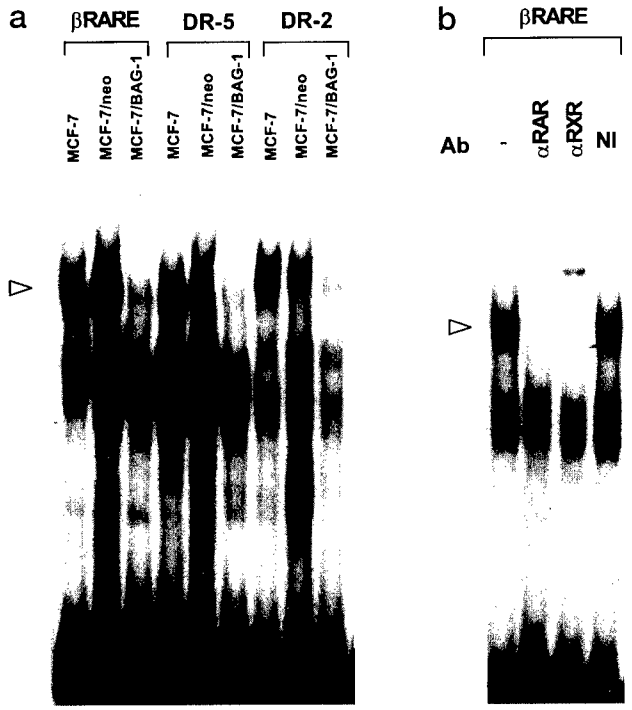

FIG. 8. **Overexpression of BAG-1 inhibits DNA binding of RAR/RXR heterodimer in MCF-7 cells.** *a*, an equal amount (3 μg) of nuclear extracts prepared from MCF-7, MCF-7/neo, and MCF-7/BAG-1(#3) was analyzed by gel retardation assay using βRARE, DR-5-type RARE (*DR-5*) or DR-2-type RARE (*DR-2*) as a probe. *b*, effects of anti-RXR (*αRXR*) and anti-RAR (*αRAR*) antibodies on the binding of the slow-migrating binding complex. Nuclear extract (3 μg) from MCF-7 cells was incubated with antibody (*Ab*) for 30 min at room temperature before performance of the gel retardation assay using the βRARE as a probe. The *arrow* indicates the slow-migrating complex, which had a binding that was inhibited by either αRXR or αRAR but not by nonspecific preimmune serum (*NI*).

DISCUSSION

Previously, it was reported that BAG-1 (RAP46) can interact with several steroid hormone receptors (43). However, the biological effects of the interaction are unknown. In this report, we show that the short BAG-1 isoform that is known to be predominantly expressed in cytoplasm (35) can antagonize RAR activity through its direct interaction with RAR. Using gel retardation assays, we observed that BAG-1 inhibits binding of RAR/RXR heterodimers to several RAREs (Fig. 1). In GST-pull down assay, we found that BAG-1 directly interacts with RAR in solution (Fig. 2). By using yeast two-hybrid assays (Fig. 3), we showed that the BAG-1-RAR interaction could occur *in vivo*. Moreover, a functional interaction was demonstrated by our observation that co-transfection of BAG-1 inhibits *trans*-RA-induced RAR transactivation activities on several RAREs (Fig. 4). Furthermore, RAR/RXR RARE binding was abrogated in MCF-7 cells that express transfected BAG-1 (Fig. 8). Thus, the interaction of BAG-1 with steroid hormone receptors can be extended to RAR. We also present evidence here that BAG-1 can similarly prevent TR DNA binding and transactivation activity. However, BAG-1 does not interact with all nuclear hormone receptors, as shown here by the failure of BAG-1 to bind to and modulate the activity of RXR (Figs. 1*d*, 2, 3, and 4*e*).

Activation or repression of gene transcription by nuclear hormone receptors requires their interaction with multiple cellular co-regulatory factors. These include receptor co-activators, which exert their effect on receptor transactivation activity by mediating transcription-initiation complex formation and affecting chromatin structure (15), and receptor co-repressors, which bind to receptors in the absence of ligand and actively repress target gene transcription by impairing the activity of the basal transcription machinery (25). BAG-1 appears to function differently from receptor co-activators or co-repressors. It does not induce ligand-dependent gene activation nor does it cause repression of target gene transcription in the absence of ligand (Fig. 4*a*). Instead, interaction of RAR and BAG-1 resulted in inhibition of RAR DNA binding (Fig. 1) and *trans*-RA-induced RAR transactivation activities (Fig. 4). Thus, it may function as a modulator of RAR activities through the mechanisms that resemble the effect of AP-1, which was previously shown to inhibit *trans*-RA-induced RAR activity by preventing RAR binding to target DNA sequences in promoters (27). However, whether BAG-1 affects recruitment of co-activator or co-repressor by RAR remains to be determined.

The mechanism by which BAG-1 inhibits RAR binding to DNA and transactivation activity remains to be elucidated. Recently, BAG-1 was reported to bind tightly with Hsp70/Hsc70-family proteins and modulate their activity (40–42). Although the role of molecular chaperones in transcriptional activation by retinoid receptors remains controversial (49, 50), Hsp70/Hsc70 and other heat shock proteins are known to participate in the regulation of several other steroid hormone receptors (44). It is therefore tempting to speculate that BAG-1 may also influence RAR activity through Hsp70/Hsc70-mediated conformational changes that prevent it from binding DNA and transactivating retinoid-responsive target genes. Interestingly, a deletion mutant of BAG-1 lacking its C-terminal 47 amino acids, which does not bind to Hsc70, was capable of

binding to RAR *in vitro* (Fig. 2). Thus, the domains in BAG-1 required for interactions with RAR and Hsp70 appear to be separable. Unfortunately, when expressed in mammalian cells, the BAG-1 mutant protein was unstable, precluding functional evaluation of its effects on RAR-mediated gene expression. Thus, the relevance of BAG-1 interactions with Hsp70-family proteins to its function as inhibitor of RAR remains to be determined.

One of the interesting features of BAG-1 is its ability to promote cell survival (34, 36, 37). The effect was previously attributed to its interaction with Bcl-2 (34). In addition, BAG-1 interacts with platelet-derived growth factor and hepatocyte growth factor receptors, enhancing their ability to transduce signals that promote cell survival (39). BAG-1 can also bind to Raf-1 and activate its kinase activity (38). Our observations that overexpression of BAG-1 inhibits *trans*-RA-induced apoptosis (Fig. 6) and prevents *trans*-RA-induced down-regulation of Bcl-2 expression (Fig. 7) suggest that interaction with RAR may represent another mechanism by which BAG-1 promotes cell survival. These observations also suggest that overexpression of BAG-1 may contribute to retinoid resistance in certain malignancies. Taken together with recent observations that BAG-1 protein levels are elevated in breast and prostate cancers (51), BAG-1 may represent an important regulator of cell survival and growth, which may contribute in multiple ways to tumorigenesis and resistance to therapy.

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Note Added in Proof—The longer isoform of BAG-1, BAG-1L, has been reported recently to enhance androgen receptor transactivation, implying that BAG-1 family proteins may either potentiate or suppress the actions of specific nuclear receptors (Froesch, B. A., Takayama, S., and Reed, J. C. (1998) *J. Biol. Chem.* **273,** 11660–11666).

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Identification of a Novel Class of Retinoic Acid Receptor β-Selective Retinoid Antagonists and Their Inhibitory Effects on AP-1 Activity and Retinoic Acid-induced Apoptosis in Human Breast Cancer Cells*

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Four candidate retinoid antagonists (LE135, LE511, LE540, and LE550) were designed on the basis of the ligand superfamily concept and synthesized. Analysis of these related retinoids by transient transfection assay demonstrated that LE135, LE540, and LE550 are effective retinoic acid receptor (RAR) antagonists, whereas LE511 selectively induced RARβ transcriptional activity. Both LE135 and LE540 inhibited retinoic acid (RA)-induced transcriptional activation of RARβ, but not RARα, RARγ or retinoid X receptor α (RXRα), on a variety of RA response elements. The retinoid antagonists also inhibited all-*trans*-RA-induced transcriptional activation of RARβ/RXRα heterodimers, although they did not show any effect on transactivation activity of RXR/RXR homodimers. In ZR-75-1 human breast cancer cells, cotreatment of LE135 and LE540 with all-*trans*-RA inhibited all-*trans*-RA-induced apoptosis of the cells, further demonstrating that RARβ plays a role in RA-induced apoptosis of breast cancer cells. We also evaluated the effect of these retinoids on AP-1 activity. Our data showed that LE135 and LE540 strongly repressed 12-*O*-tetradecanoylphorbol-13-acetate-induced AP-1 activity in the presence of RARβ and RXRα. Interestingly, LE550 induced AP-1 activity when RARβ and RXRα were expressed in HeLa cells but not in breast cancer cells. These results demonstrate that LE135 and LE540 were a novel class of RARβ-selective antagonists and anti-AP-1 retinoids and should be useful tools for studying the role of retinoids and their receptors.

Retinoids, the natural and synthetic vitamin A derivatives, are known to regulate many biological processes, including growth, differentiation, and development (1–3). They are currently used in the treatment of epithelial cancer and promyelocytic leukemia and are being evaluated as preventive and therapeutic agents for a variety of other human cancers (4). One of the major drawbacks of retinoid therapy has been the wide range of undesirable side effects. Development of anticancer-specific retinoids with improved clinical value is largely dependent on the understanding of the mechanistic basis of the pleiotropic activities induced by retinoids and their receptors. The effects of retinoids are mainly mediated by two classes of nuclear retinoid receptors: the retinoic acid receptors (RARs)[1] (5–9) and the retinoid X receptors (RXRs) (10–14), and both receptors are members of the steroid-thyroid hormone receptor superfamily and are encoded by three distinct genes, α, β, and γ (5–14). All-*trans*-retinoic acid (RA) acts as a ligand for RARs, while 9-*cis*-RA is a ligand for both RARs and RXRs. RARs and RXRs modulate the expression of their target genes by interacting as either homodimers or heterodimers with RA response elements (RAREs) (11, 14–17). A RARE (βRARE) in the RARβ gene promoter mediates RA-induced RARβ gene expression in many different cell types (18, 19). Up-regulation of the RARβ gene by RA plays a critical role in amplifying the RA response and is required for RA-induced growth inhibition and apoptosis in human breast cancer (20) and lung cancer cells (21).

In addition to the regulation of RARE-containing genes, retinoid receptors can inhibit the effect of the tumor promoter TPA by repressing the transcriptional activity of AP-1 (22). Inhibition of AP-1 activity by retinoid receptors may involve either direct protein-protein interaction between retinoid receptor and components of AP-1, such as c-Jun (23), or competition for a common coactivator CBP (24). Recent studies have suggested that different receptor conformational changes may account for gene regulation on RAREs and inhibition of AP-1 activity and that they can be dissociated as anti-AP-1-specific retinoids have been described (25–27). Interaction between membrane and nuclear receptor signaling pathways mediated by RAR/AP-1 interaction may represent an important mechanism underlying the potent antineoplastic effects of retinoids. Because many of the AP-1 responsive genes, such as collagenases and stromelysins, are involved in cancer cell proliferation and transformation (28), retinoids that specifically inhibit AP-1 activity may be therapeutically desirable because they may have reduced side effects associated with gene activation, but retain their anticancer activity. This is demonstrated by recent studies showing that retinoids that inhibit AP-1 activity but cannot induce transactivation of RARE-containing genes were able to inhibit TPA-induced transformation and the clonal growth of mouse epidermal JB6 cells (29). In addition, a group of anti-AP-1-specific retinoids inhibited the proliferation of lung and breast cancer cells but had impaired ability to induce differentiation of F-9 cells (25).

Each subtype of RARs has been implicated in the regulation of cancer development and the anticancer activities of retinoids. Translocation of the RARα gene is responsible for the development of acute promyelocytic leukemia (30), whereas RARγ may play a role in mediating growth inhibition and

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[1] The abbreviations used are: RAR, retinoic acid receptor; RA, retinoic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; RXR, retinoid X receptor; RARE, RA response element.

apoptosis by certain retinoids (31). Recently, evidence is emerging showing that RARβ may play a critical role in the regulation of cancer cell growth. RARβ is located in chromosome 3p24, a region that is often deleted or mutated in a variety of cancer (32), and RARβ was found to be integrated by hepatitis B virus in human liver cancer (33). It is not expressed in many different types of cancer cell lines (20, 34–38), and re-expression of RARβ in RARβ-negative cancer cells restored the ability of RA to induce growth inhibition and apoptosis (20). Despite these studies, further investigation is needed to dissect function of each retinoid receptor in cancer cells. Several approaches have been used often to determine specific function of each receptor subtype, including loss of function, such as knock-out and antisense technique, and gain-of-function, such as ectopic expression of a receptor subtype. RAR subtype-selective agonists and antagonists are being developed (39–43), and they have been widely used to study the function of each receptor. However, the degree of selectivity and receptor transactivation activity needs to be improved. In addition, development of more and effective RARβ-selective retinoids is important to further study RARβ function in cancer. Furthermore, receptor-selective retinoids allow dissociation of desired and undesired effects of retinoids and are also believed to be more specific and with less toxicity in cancer prevention and treatment.

To further study RARβ function in cancer cells, we analyzed a class of conformational restricted retinoids. Our data demonstrated that LE135, LE540, and LE550 inhibited all-*trans*-RA-induced transcriptional activation of RARβ but not RARα, RARγ, or RXRα on a number of RAREs, whereas LE511 selectively induced transactivation activity of RARβ. The RARβ-selective antagonist effect was further demonstrated by their ability to inhibit all-*trans*-RA-induced apoptosis of ZR-75-1 human breast cancer cells. Interestingly, the antagonists LE135 and LE540 also exert anti-AP-1 activity. They effectively repressed TPA-induced AP-1 activity in both HeLa and breast cancer cells when RARβ was expressed. In contrast, LE550 induced AP-1 activity in HeLa but not in breast cancer cells. Together our results demonstrate that a novel class of RARβ-selective retinoids with variable biological functions should represent useful tools for studying RARβ function.

EXPERIMENTAL PROCEDURES

Retinoids—All-*trans*-RA was obtained from Sigma. LE135 was prepared as described by Eyrolles *et al.* (44). LE511, LE540, and LE550 were prepared as described by (45). Ro 41-5253 was kindly provided by Dr. Michael Klaus (46).

Cell Culture—Monkey kidney CV-1 and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, and ZR-75-1 breast cancer cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum.

Plasmid Constructions—The receptor expression plasmids pECE-RARα, -RARβ, -RARγ, and -RXRα and the construction of the reporter plasmids βRARE-tk-CAT, TREpal-tk-CAT, CRBPI-RARE-tk-CAT, and ApoAI-RARE-tk-CAT have been described previously (15, 16, 47, 48).

Transient Transfection Assay—CV-1 cells were seeded at 5.0×10^5 cells/well in 24-well plates. A modified calcium phosphate precipitation procedure was used for transient transfection as described previously (20). 100 ng of reporter plasmid, 100 ng of β-galactosidase expression vector (pCH110, Amersham Pharmacia Biotech), and various amounts of receptor were mixed with carrier DNA (pBluescript, Stratagene) to 1,000 ng of total DNA/well. After 16–24 h, transfected cells were treated with or without 10^{-7} M all-*trans*-RA in the absence or presence of the indicated concentrations of retinoid antagonists. For anti-AP-1 assay, a reporter construct containing the collagenase promoter linked with the CAT gene, -73-Col-CAT (23), was used in HeLa and ZR-75–1 cells. After transfection, cells were grown in a medium containing 0.5% charcoal-treated fetal calf serum with retinoids and/or TPA (100 ng/ml). Transfection efficiency was normalized to β-galactosidase activity. The data shown are the means of three separate experiments.

Apoptosis Assay—Nuclear morphological change analysis and DNA fragmentation (TdT) assay were as described previously (20, 21). For nuclear morphological analysis, ZR-75-1 cells were treated with or without 10^{-7} M all-*trans*-RA in the absence or presence of 10^{-6} M LE135, LE540, or LE550 for 4 days, trypsinized, washed with phosphate-buffered saline, fixed with 3.7% paraformaldehyde, and stained with 50 μg/ml 4,6-diamidino-2-phenylindole containing 100 μg/ml DNase-free RNase A to visualize the nuclei. Stained cells were examined by fluorescent microscopy. For DNA fragmentation (TdT) assay, ZR-75-1 cells were treated with or without 10^{-7} M all-*trans*-RA in the absence or presence of 10^{-6} M LE135, LE540, or LE550. After 3 days, cells were trypsinized, washed with phosphate-buffered saline, fixed in 1% formaldehyde in phosphate-buffered saline, washed with phosphate-buffered saline, resuspended in 70% ice-cold ethanol, and immediately stored at −20 °C overnight. Cells were then labeled with biotin-16-dUTP by terminal transferase and stained with avidin-fluorescein isothiocyanate (Roche Molecular Biochemicals). The labeled cells were analyzed using a FACScater-Plus. Representative histograms are shown.

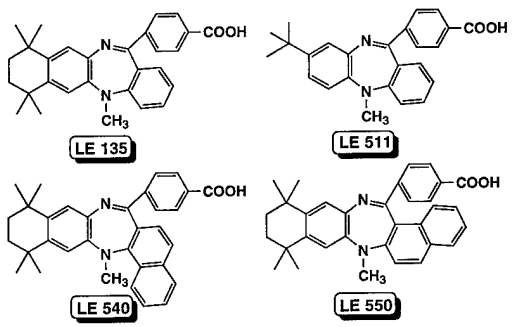

FIG. 1. **Chemical structure of the synthetic retinoids.**

RESULTS

Transactivation Activity of the Synthetic Retinoids—Recently, we designed several retinoid antagonists, LE135, LE511, LE540, and LE550 (49, 50), based on the structure-activity relationship of retinoids (Fig. 1). Transcriptional activation of these retinoids on RARα, RARβ, RARγ, or RXRα was determined by transient transfection assay using the reporter construct TREpal-tk-CAT (15) in CV-1 cells. As shown in Fig. 2, all-*trans*-RA strongly induced transcriptional activation of each RARs, whereas 9-*cis*-RA effectively promoted RXR homodimer activity. However, LE135, LE540, and LE550, at 10^{-7} M and 10^{-6} M, had very little effect on transcriptional activation of RARα, RARβ, RARγ, or RXRα. LE135 and LE540 even showed inhibitory effect on basal RARβ activity in a dose-dependent manner. Interestingly, LE511, a LE135 analog with bulky acyclic alkyl group, showed a strong induction of RARβ transactivation activity. Activation of RARβ could be observed when 10^{-7} M LE511 was used, and it is about 65% of efficiency as compared with all-*trans*-RA. Together, these results demonstrate that LE135, LE540, and LE550 are ineffective on transcriptional activation of RARs and RXR, whereas LE511 is a RARβ-selective agonist.

Antagonistic Effect of the Synthetic Retinoids on RA-induced RARβ Transcriptional Activation—LE135 was previously shown to selectively bind to RARβ (44). The observations that LE135 could not induce RARβ transactivational activity and that it inhibited basal RARβ activity (Fig. 2) suggested that binding of RARβ by LE135 might repress its transactivation function. We therefore analyzed the effect of LE135 on all-*trans*-RA-induced transcriptional activation of RARβ on βRARE-tk-CAT reporter (19). For comparison, its effect on RARα or RARγ was analyzed. As shown in Fig. 3*a*, 10^{-7} M all-*trans*-RA-induced RARβ activity was strongly inhibited by LE135 in a concentration-dependent manner, with more than 70% inhibition when 10^{-6} M LE135 was used. For comparison, RARα-selective antagonist Ro 41-5253 did not show any effect on RARβ activity. When RARα was analyzed, LE135 did not

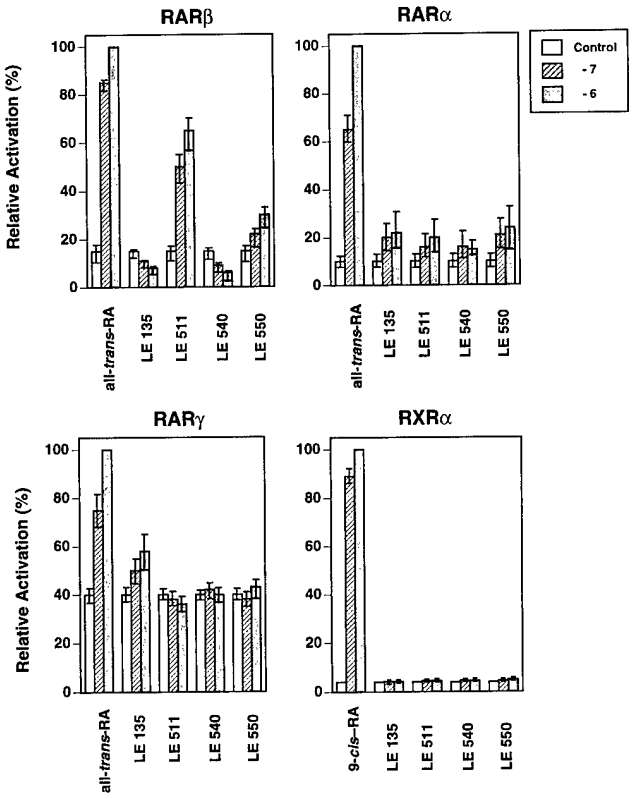

FIG. 2. **Transcriptional activation profiles.** Transcriptional activation activities of retinoids were determined on RARα, RARβ, RARγ, or RXRα receptors. CV-1 cells were transiently transfected with 100 ng of TREpal-tk-CAT reporter plasmid and 100 ng of RARα, RARβ, or RARγ or 20 ng of RXRα receptor expression plasmid. Transfected cells were grown in the absence or presence of the indicated concentrations of retinoids, and assayed for CAT activity after 24 h. 100% activity was the reporter gene activity measured in the presence of 10^{-6} M all-*trans*-RA for RARs or 10^{-6} M 9-*cis*-RA for RXRα after subtraction of constitutive receptor activity.

exhibit clear inhibitory effect on all-*trans*-RA-induced RARα activity, whereas Ro 41-5253 significantly repressed the reporter transcription. Both LE135 and Ro 41-5253 did not show any influence on all-*trans*-RA-induced RARγ activity. Similar results were obtained on another reporter, the TREpal-tk-CAT (Fig. 3*b* and data not shown). Because RARβ is likely to function as RARβ/RXR heterodimer in cells, we determined whether LE135 could act as a RARβ/RXR heterodimer antagonist. As shown in Fig. 3*b*, LE135 exhibited a similar degree of inhibition on both all-*trans*-RA-induced RARβ and RARβ/RXR heterodimer activity (Fig. 3*b*), whereas it had no effect on 9-*cis*-RA-induced RXR homodimer activity. Similar results were obtained with LE540 (data not shown). Together, these data demonstrate that LE135 and LE540 are effective antagonist of RARβ and RARβ/RXR heterodimer.

To further analyze the antagonist effect of LE135 and its analogs, reporter constructs containing different RAREs, including βRARE, CRBPI-RARE and ApoAI-RARE were used. As shown in Fig. 3*c*, LE135, LE540, and LE550 inhibited all-*trans*-RA-induced RARβ transcriptional activation on these different RAREs. Similar degrees of inhibition (about 60–70%) were observed with the βRARE and the ApoAI-RARE, whereas a less degree of inhibition (50–60%) was obtained with the CRBPI-RARE. Interestingly, LE540 showed a more effective inhibition on all RAREs than its isomer LE550, consistent with their antagonist effect on HL-60 cell differentiation (45). These results demonstrate that LE135, LE540, and LE550 could inhibit transactivation of RARβ and that the antagonistic effect of these retinoids is response element independent.

Effect of the Retinoid Antagonists on RA-induced Growth Inhibition and Apoptosis in Human Breast Cancer Cells—We have previously demonstrated that expression of RARβ is required for all-*trans*-RA-induced apoptosis of human breast cancer cells (20). We then analyzed whether inhibition of RARβ activity by RARβ-selective antagonists could repress all-*trans*-RA activity in ZR-75-1 human breast cancer cells. ZR-75-1 cells underwent extensive apoptosis when they were treated with all-*trans*-RA as revealed by both morphological analysis (DAPI staining) (Fig. 4*a*) and DNA end-labeling assay (TdT) (Fig. 4*b*). However, all-*trans*-RA-induced apoptosis was strongly prevented when 10^{-7} M all-*trans*-RA was used together with 10^{-6} M of LE135, LE540, or LE550. Morphological analysis showed that all-*trans*-RA-induced apoptosis was reduced from about 40 to 20% by LE135, LE540, or LE550 (Fig. 4*a*). Similar results were obtained by TdT assay (Fig. 4*b*). These results are in agreement with previous observation made by ectopic expression of RARβ (20), and suggest that these RARβ-selective antagonists are useful tools for studying RARβ function.

Effect of Retinoid Antagonists on TPA-induced AP-1 Activity—Recently, it was reported that RA can inhibit AP-1 activity (22) and that receptor conformational change required for AP-1 inhibition is different from that required for receptor transcriptional activation and they can be dissociated (25–27). To determine whether LE135, LE540, and LE550 could also induce RAR conformational changes for inhibiting AP-1 activity, we investigated their effect on TPA-induced AP-1 activity. When a reporter containing the collagenase (23) linked with the CAT gene, -73Col-CAT (23), was transiently transfected into HeLa cells, treatment of the cells with TPA strongly induced the reporter activity, consistent with previous observations. Cotreatment of the cells with either all-*trans*-RA, LE135, LE540, or LE550 did not show a clear effect on TPA-induced reporter activity (Fig. 5*a*). However, when RARβ was cotransfected, the TPA-induced reporter transcription was slightly inhibited, which was further inhibited when cells were treated with all-*trans*-RA but not with LE135, LE540, and LE550. Interestingly, treatment with LE550 slightly enhanced reporter transcription. When RARβ and RXRα were cotransfected, however, LE135 and LE540 showed a strong inhibition of the TPA-induced reporter activity. The enhancing effect of LE550 was also increased. These data suggest that LE135 and LE540 could induce a conformational change of RARβ required for inhibiting AP-1 activity only when RARβ is heterodimerized with RXRα and that LE550 may induce another RARβ conformation that stimulates AP-1 transcriptional activity. To determine whether these retinoids could inhibit AP-1 activity in breast cancer cells, the -73Col-CAT was transiently transfected into ZR-75-1 cells (Fig. 5*b*). Treatment of the cells with TPA led to an increase of reporter gene transcription for about 7-fold. Both LE135 and LE540 inhibited the TPA-induced activity in a concentration-dependent manner when RARβ expression vector was cotransfected. These data demonstrate that LE135 and LE540 could also inhibit AP-1 activity in breast cancer cells. The fact that LE135 and LE540 could inhibit the TPA-induced reporter transcription without RXRα cotransfection is consistent with observation that RXRα is expressed in ZR-75-1 cells (20). Interestingly, we did not observe any induction of the reporter activity when cells were treated with LE550, suggesting that the AP-1-inducing effect of LE550 is cell-type specific.

DISCUSSION

Previous studies on receptor-ligand interaction have suggested requirements for being a potent retinoid antagonist: (i) strong binding to the receptor mediated by the hydrophobic

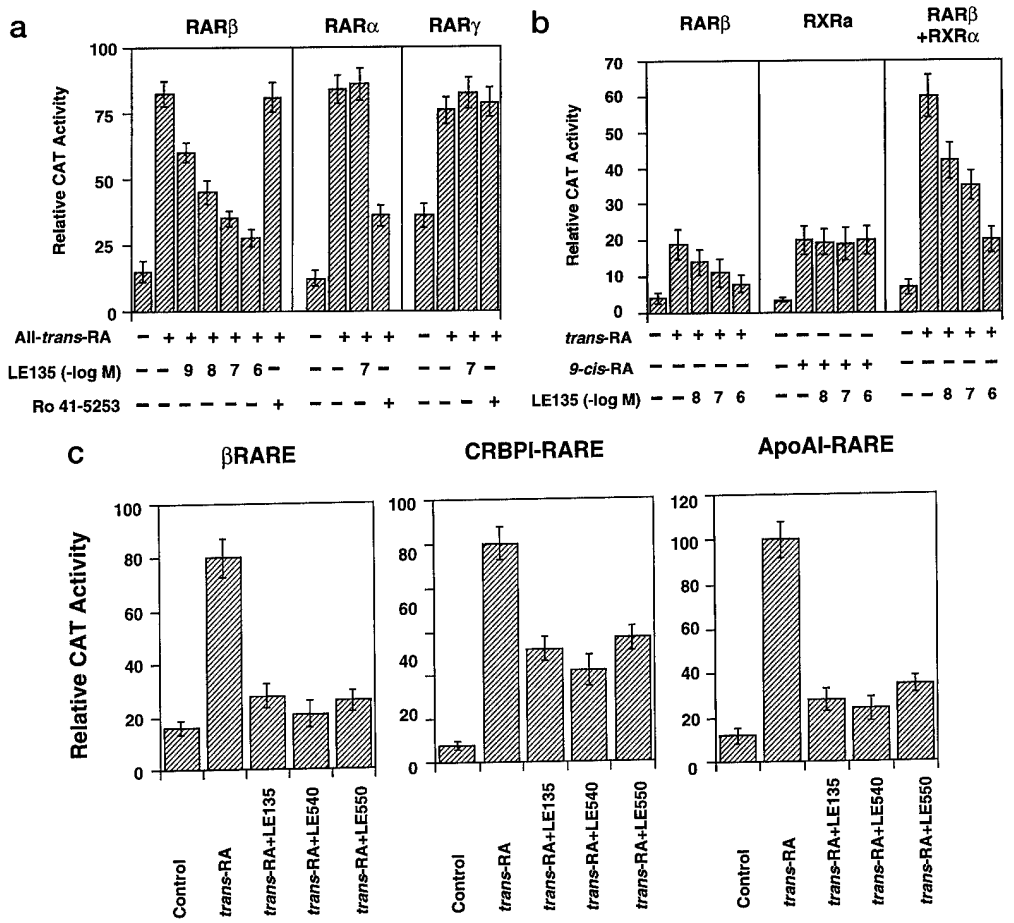

FIG. 3. **Antagonistic effects of retinoids on RA-induced transcriptional activation of the RARs.** *a*, effect of LE135 on RARα, RARβ, or RARγ receptor on βRARE. RARα, RARβ, or RARγ expression vector (100 ng) was cotransfected with the βRARE-tk-CAT repoter into CV-1 cells. Transfected cells were treated with 10^{-7} M all-*trans*-RA in the absence or presence of the indicated concentration of LE135 or Ro 41-5253 (10^{-7} M). *b*, effect of LE135 on RARβ, RXRα, or RARβ/RXRα heterodimer activity on the TREpal. RARβ (100 ng), RXRα (25 ng), or RARβ/RXRα expression vectors were cotransfected with the TREpal-tk-CAT reporter into CV-1 cells. Transfected cells were treated with 10^{-7} M all-*trans*-RA in the absence or presence of the indicated concentrations of LE135. *c*, the antagonist effect of retinoids is response element independent. CV-1 cells were cotransfected with 100 ng of RARβ expression plasmid and 100 ng of βRARE-tk-CAT, CRBPI-RARE-tk-CAT or ApoAI-RARE-tk-CAT reporter. Transfected cells were treated with 10^{-7} M all-*trans*-RA in the absence or presence of the 10^{-6} M LE135, LE540, or LE550.

alkylated benzo group of a retinoid contributes to its binding affinity to RARs and (ii) inhibition of the conformational changes, such as proper folding of the helix 12 where the binding site for co-activators (AF2 domain) exists. This has established structure-activity relationships in both retinoid agonists and antagonists. We describe here a class of related synthetic retinoids, LE135, LE540, LE511, and LE550, which either specifically activate or inhibit RARβ transactivation function. In assaying for transactivation function of the retinoids on each subtype of RARs and RXRα, LE511 selectively activated RARβ (Fig. 2), suggesting that it is a RARβ-selective agonist. In contrast, other LE compounds, LE135, LE540, and LE550, did not display any activation function on the retinoid receptors tested (Fig. 2). Instead, LE135 and LE540 showed a strong inhibition of RARβ basal transactivation activity. When they were used together with all-*trans*-RA, LE135 or LE540 effectively inhibited all-*trans*-RA-induced RXRα/RARβ activity (Fig. 3). LE135 does not bind to RXRs and RARγ in receptor binding assay (45). It binds with higher affinity to RARβ (K_i = 0.22 μM) than to RARα (K_i = 1.4 μM) (45). Thus, LE135 and its related analog LE540 act as RARβ-selective antagonists. These results suggest that the tetramethyltetrahydronaphthalenyl group as seen in LE135, LE540, and LE550 may be required for RARβ antagonist effect, because its replacement with effective mono-tertiary butylphenyl group as seen in LE511 resulted in loss of RARβ antagonism effect (Fig. 3). The smaller hydrophobic *tert*-butyl group of LE511 not only decreases the binding affinity to RARs but also may change the binding occupation in the ligand-RAR complex with less disturbance of the helix folding. This may explain the critical agonistic activity of LE511. The existence of another benzo (or naphtho) group impairs the transcriptional activating activity due to the different conformational change in the ligand-receptor complex. From the reported crystal structures of RXRα and RARγ (51, 52), proper folding of the helix 12 in the ligand-binding domain of RARs is critical for receptor activation. The benzo/naphtho group fused to the diazepine ring may disturb the proper folding of the helix to elicit the antagonistic activity. The bulkier naphtho group of LE540 is expected to be more effective than the benzo group of LE135 and that of LE550 with a different direction seems to affect weakly the conformation around the helix as it could not inhibit RARβ basal transactivation activity (Fig. 2).

The pleiotropic effects of retinoids are mainly mediated by RARs and RXRs. Both types of retinoid receptors are encoded by three distinct genes, α, β, and γ. The fact that these receptors display distinct patterns of expression during development and differentiation suggests that each of them may have specific function, which is being unraveled recently by a variety of technologies, such as homologous recombination, antisense, and ectopic expression of a receptor of interest. The complexity of retinoid responses also can be dissected with the use of both

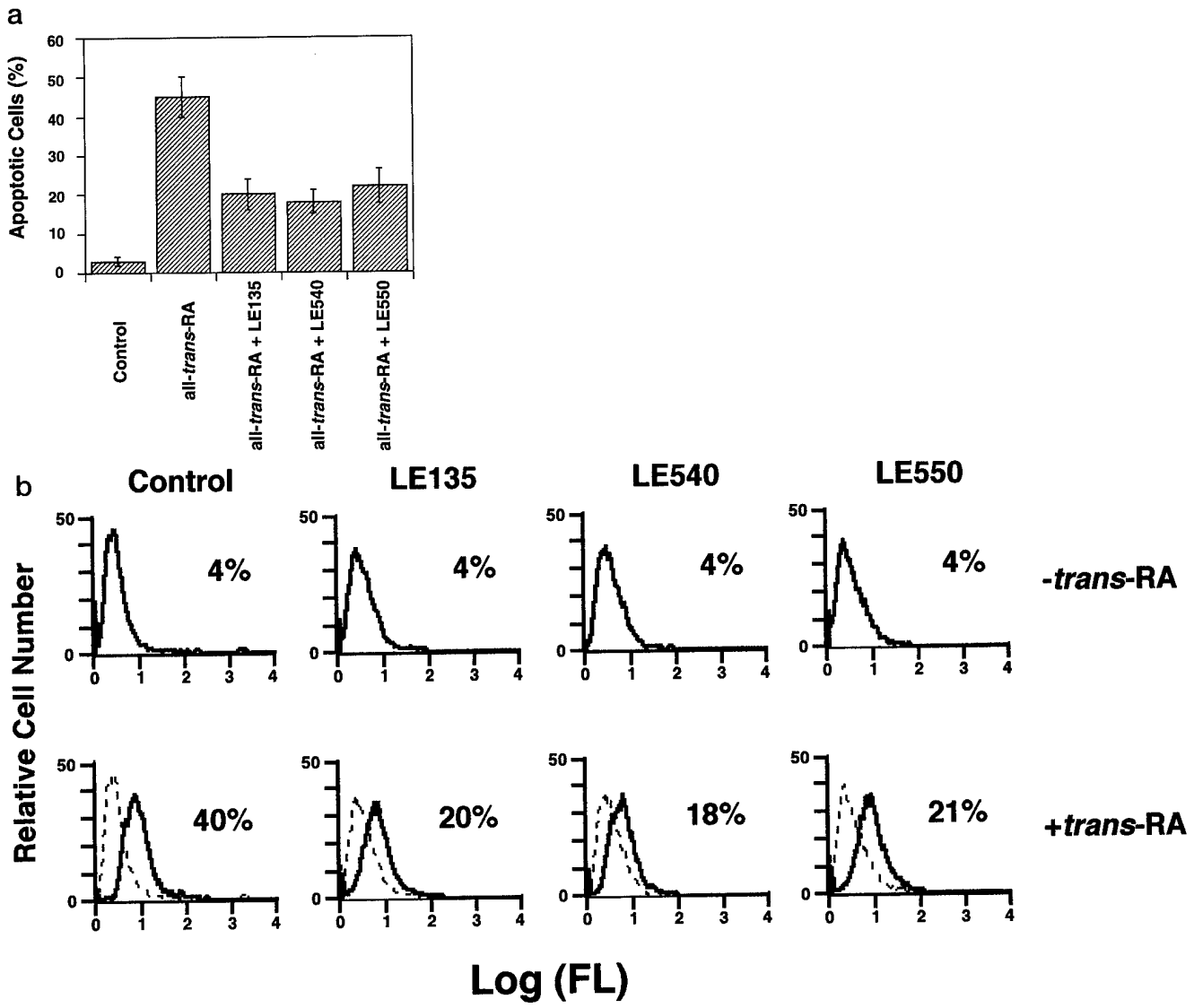

FIG. 4. **Effect of the retinoid antagonists on RA-induced apoptosis in ZR-75-1 human breast cancer cells.** *a*, morphological analysis of apoptotic breast cancer cells. ZR-75-1 cells were treated with or without 10^{-7} M all-*trans*-RA in the absence or presence of 10^{-6} M LE135, LE540, or LE550 for 4 days and nuclear morphology was analyzed by DAPI staining. *b*, DNA fragmentation analysis. ZR-75-1 cells were treated with or without 10^{-7} M all-*trans*-RA in the absence or presence of 10^{-6} M LE135, LE540, or LE550 for 3 days, and DNA fragmentation was determined by the TdT assay. Representative histograms show relative apoptotic cell numbers. *FL*, fluorescence.

receptor-selective agonist and antagonists, activating or interfering specifically or preferentially with one given receptor. We have previously reported (20) that all-*trans*-RA-induced apoptosis of human breast cancer cells requires RARβ expression. This was based on our observation that stable expression of RARβ in RARβ-negative cells induced apoptosis, whereas expression of RARβ antisense RNA in RARβ-positive cells abolished apoptotic effect of all-*trans*-RA (20). Here, we used RARβ-selective antagonists to study the involvement of RARβ in all-*trans*-RA-induced growth inhibition and apoptosis of ZR-75-1 human breast cancer cells. When RARβ-selective antagonists LE135, LE540, or LE550 was used together with all-*trans*-RA, the effect of all-*trans*-RA on apoptosis in ZR-75-1 cells was largely reduced (Fig. 4). This result further supports the role of RARβ in all-*trans*-RA-induced growth inhibition and apoptosis of human breast cancer cells. It also demonstrates that LE135 and its analogs are valuable tools for studying RARβ function.

In this study, we also show that RARβ antagonists could act as effective anti-AP-1 retinoids (Fig. 5). These retinoids, which did not show any transactivation function on RARs and RXRs, could repress AP-1 activity in the presence of RARβ/RXR heterodimer. Therefore, they are anti-AP-1 specific retinoids. Interestingly, they could not affect AP-1 activity in the presence of RARβ alone but required RXR for effective inhibition of AP-1 activity. This suggests that binding of retinoids to RARβ alone is not sufficient to induce a specific anti-AP-1 conformational change. Interestingly, LE550 could induce AP-1 activity in HeLa cells but not in ZR-75-1 cells (Fig. 5). The mechanism by which LE550 induces AP-1 activity is unclear. It is likely that LE550 induces a different conformational change of RARβ. This is supported by our observation that LE540 and LE135 inhibited basal RARβ activity, whereas LE550 could not (Fig. 2). This is interesting since LE540 and LE550 are regio-isoforms, in which the bulkier naphtho group of both compounds is arranged in different directions, which may be critical for AP-1 interaction (Fig. 5). Previous studies have demonstrated that AP-1 can either inhibit or stimulate nuclear receptor activity, depending on cell type, promoter, and nuclear receptor (53). Our observation that LE550 could induce AP-1 activity in HeLa cells suggests that the retinoid receptor could also stimulate AP-1 activity in response to appropriate ligand. The fact

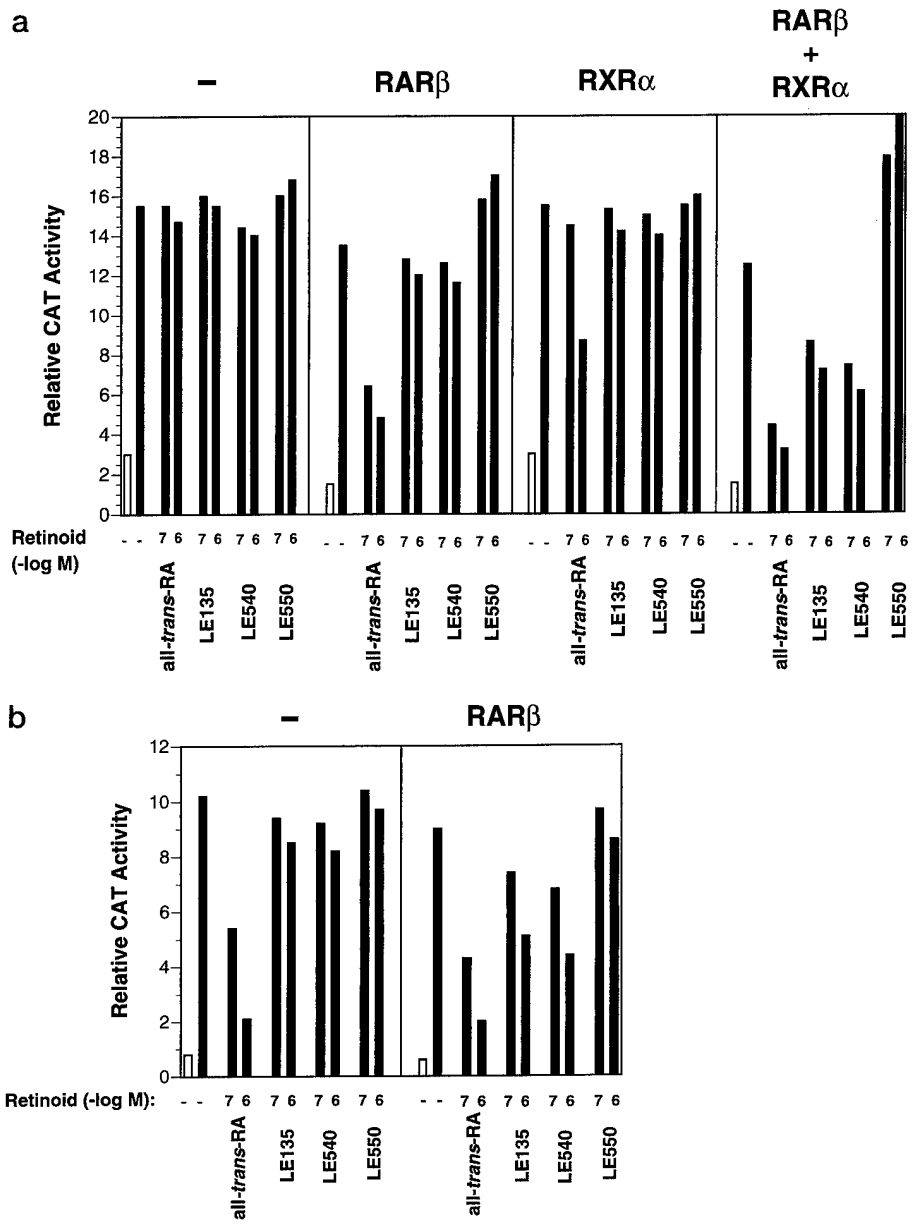

FIG. 5. **Effect of retinoids on TPA-induced collagenase promoter activity.** *a*, effect of LE compounds on TPA-induced collagenase promoter activity in HeLa cells. The -73Col-CAT reporter (100 ng) was cotransfected without or with the indicated retinoid receptor expression vector (100 ng) into HeLa cells. After transfection, cells were cultured in Dulbecco's modified Eagle's medium containing 0.5% FCS and treated with either all-*trans*-RA or the indicated retinoids and/or TPA (100 ng/ml). 24 h later, the cells were harvested and CAT activities were determined. The mean of CAT activity in three independent experiments is shown. *b*, effect of LE compounds on TPA-induced collagenase promoter activity in ZR-75-1 cells. The -73Col-CAT reporter (250 ng) was transfected into ZR-75-1 cells and assayed for its activity as described in *panel a*. *Empty bar*, no TPA treatment; *black bar*, with TPA treatment.

that induction of AP-1 activity by LE550 was only observed in HeLa cells but not in ZR-75-1 cells implies that inhibition or induction of AP-1 activity by retinoid receptor is also cell-type specific. LE550 may induce RARβ in a conformation that allows a positive effect on AP-1 transcription, probably through transcriptional mediators specifically expressed in HeLa cells. Such a compound may be a valuable tool for studying mechanisms underlying AP-1/nuclear receptor interaction and for dissecting complexity of AP-1/nuclear receptor interaction.

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Differential Effect of Retinoic Acid on Growth Regulation by Phorbol Ester in Human Cancer Cell Lines*

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Phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and all-*trans*-retinoic acid (*trans*-RA) are potent regulators of growth of cancer cells. In this study, we investigated the effect of TPA and *trans*-RA alone or their combination on proliferation of human breast cancer ZR75-1 and T47D and lung cancer H460 and H292 cell lines. *trans*-RA caused various degrees of growth inhibition of these cell lines. However, TPA showed inhibition of proliferation of H460 and H292 cells and induction of ZR75-1 cell growth. Although *trans*-RA did not significantly regulate the growth inhibitory effect of TPA, it completely prevented its growth stimulating function. The divergent effects of TPA were associated with specific disruption of cell cycle events, an induction of G_0/G_1 arrest in H460 and H292 cells and inhibition of G_0/G_1 arrest with increase of S phase in ZR75-1 cells. Induction of G_0/G_1 arrest was accompanied by induction of $p21^{WAF1}$ and ERK activity, whereas inhibition of G_0/G_1 arrest was associated with enhanced activity of JNK and AP-1 but not ERK. *trans*-RA did not affect TPA-induced $p21^{WAF1}$ expression. However, it inhibited TPA-induced AP-1 activity in ZR75-1 cells and the constitutive AP-1 activity in H460 and H292 cells. Thus, *trans*-RA modulates TPA activity through its interaction through TPA-induced JNK/AP-1 pathway but not TPA-induced ERK/$p21^{WAF1}$ pathway.

Phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA)[1] is a potent regulator of growth of many different cell types (1). It activates protein kinase C, which plays a key role in the control of many signal transduction pathways involved in different cellular functions, such as growth, differentiation, and cell transformation (2–4). Protein kinase C overexpression is associated with increased tumorigenicity and metastatic potential in several experimental models (5), and its activity is increased in tumors of breast and lung as compared with their normal counterparts (6). Activation of protein kinase C by TPA can lead to growth stimulation and cellular transformation (5–7) and is in part due to induction of AP-1, a collection of sequence-specific transcriptional activators composed of members of the c-Jun and c-Fos families, which are often associated with proliferation of cancer cells (8). TPA could also induce growth arrest and differentiation in certain leukemia cells and cancer cells, which is accompanied by induction of $p21^{WAF1}$ (9–11). Recent studies have demonstrated that induction of $p21^{WAF1}$ depends on Raf/ERK signaling and involves transcriptional activation of the $p21^{WAF1}$ promoter in a p53-independent manner (12). $P21^{WAF1}$ is believed to inhibit cell cycle progression through its interaction with cyclin-dependent kinase complexes, which are required for various cell cycle transitions (13, 14). Thus, TPA can either stimulate or inhibit cell proliferation, depending on cell type.

All-*trans*-retinoic acid (*trans*-RA) and its natural and synthetic derivatives (retinoids) regulate a broad range of biological processes, including growth, differentiation, and development in both normal and neoplastic cells (15, 16). The effect of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors and retinoids X receptors, that are encoded by three distinct genes, (α, β, and γ) and are members of the steroid/thyroid hormone receptor superfamily (17–19). Retinoid receptors modulate the expression of their target genes in response to their natural ligands *trans*-RA and 9-*cis*-RA by interacting as either homodimers or heterodimers with RA response elements. A number of RA target genes have been identified and many of them are associated with cell proliferation, differentiation, and growth (17–19).

In addition to transactivation function, retinoid receptors exert potent *trans*-repression function, which also plays an important role in mediating the diverse function of retinoids. Retinoid receptors, in response to their ligands, can inhibit the effect of TPA by repressing the transcriptional activity of AP-1 (20). The mechanism by which ligand-activated retinoid receptors repress AP-1 activity remains largely unknown, although a direct protein-protein interaction between retinoid receptors and AP-1 (20) and a competition for a common coactivator (21) have been proposed. Nevertheless, the interaction between membrane and retinoid receptor signaling pathways may represent an important mechanism by which retinoids exert their potent anti-neoplastic effect.

To further understand the growth regulatory effect of TPA and its interaction with retinoid signaling, we evaluated the interaction of TPA and *trans*-RA on growth of several human lung cancer and breast cancer cell lines and the underlying molecular mechanisms. Our results demonstrated that TPA exhibited different effects on growth of these cancer cell lines. TPA induced growth arrest of lung cancer cell lines H460 and H292 through either induction of $p21^{WAF1}$ expression and ERK activity and/or inhibition of Cdk2 expression. In contrast, TPA enhanced proliferation of ZR75-1 breast cancer cells through induction of JNK and AP-1 activity. When the effect of

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[1] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ERK, extracellular signal-regulated kinase; *trans*-RA, all-*trans*-retinoic acid; FCS, fetal calf serum; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; JNK, c-Jun N-terminal kinase.

trans-RA on TPA activity was studied, we observed that it could additively increase the growth inhibitory effect of TPA in lung cancer cells, mainly due to its repression of constitutive AP-1 activity in the cells rather than its modulation of TPA-induced $P21^{WAF1}$ expression and ERK activity. In contrast, *trans*-RA abolished the growth-stimulatory effect of TPA by repressing TPA-induced AP-1 activity in a JNK-independent mechanism in breast cancer cells. These results demonstrate that two potent growth regulators, TPA and *trans*-RA, play a critical role in regulating cancer cell growth and that *trans*-RA modulates TPA activity through its interaction with TPA-induced JNK/AP1 pathway but not TPA-induced ERK/$p21^{WAF1}$ pathway.

EXPERIMENTAL PROCEDURES

Cell Culture—The non-small cell lung cancer cell lines H460 and H292 and breast cancer cell lines ZR75-1 and T47D were obtained from American Type Culture Collection (ATCC). They were grown in RPMI 1460 medium supplemented with 10% fetal calf serum (FCS).

Growth Inhibition Assay—Cells were seeded at a density of 1,000 cells per well in 96-well plates. One day later, the desired volume of TPA was added to the cells to achieve a final concentration of 0.001–10 nM. The effect of 10^{-6} M *trans*-RA was analyzed alone or in combination with various TPA concentrations. The control cells received vehicle (ethanol). Media and retinoids were changed every 48 h. Viable cell number was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (22).

Flow Cytometry Analysis—Cells were trypsinized and collected by centrifugation at 2,000 rpm for 5 min. The cell pellets were then resuspended in 1 ml PBS and fixed in 70% ice-cold ethanol and kept in a freezer overnight. Fixed cells were centrifuged, washed once in PBS, and then resuspended in 100 μl of phosphate-citrate buffer (192 parts of 0.2 M Na_2HPO_4 and 8 parts of 0.1 M citric acid, pH 7.8) for 30 min at room temperature to wash out any degraded DNA from apoptotic cells. The cells were then collected by centrifugation at 2,000 rpm, and the cell pellets were washed twice with PBS and resuspended in PBS containing 50 μg/ml propidium iodide (Sigma) and 100 μg/ml DNase-free RNase A (Roche Molecular Biochemicals). The cell suspension, protected against light, was incubated for 30 min at 37 °C and then analyzed using the FACScater-plus Flow cytometer.

RNA Preparation and Northern Blot Analysis—For Northern blot analysis, total RNAs were prepared by the guanidine hydrochloride/ultracentrifugation method (22). About 30 μg total of RNAs from different cell lines were fractionated on 1% agarose gels, transferred to nylon filters, and probed with the ^{32}P-labeled probe as described previously (22).

Antibodies and Western Blot Analysis—Cells were lysed in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, and protease inhibitors. Aliquots containing 50 μg of proteins were resolved on 12% by SDS-polyacrylamide gel electrophoresis, followed by electrotransfer to nitrocellulose membrane. Immunodetection was carried out using anti-$p21^{WAF1}$ (Santa Cruz), anti-p53 (Oncogene Inc.), and anti-Cdk2 (Santa Cruz) antibodies in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20), followed by horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech). Detection was performed with an enhanced chemilumienescence detection kit (ECL, Amersham Pharmacia Biotech). Anti-α-tubulin antibody (Sigma) was used as a control for protein loading.

Protein Kinase Assays—Cells were seeded in six-well plates 2 days prior the analysis to provide ~80% confluent preparations. After treatment with different agents, cells were washed twice with ice-cold PBS solution and suspended in lysis buffer (25 mM HEPES, pH 7.7, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4). The Jun kinase assay was performed according to the method described previously (23). Briefly, 50 μg of whole cell lysate were mixed with 10 μg of glutathione *S*-transferase-c-Jun (1–223) (GST-c-Jun) and rotated for 3 h at 4 °C. GST-c-Jun proteins were purified from *Escherichia coli* and bound to agarose beads (Sigma). The beads were then washed twice and incubated with 20 μl of kinase reaction buffer (20 mM HEPES, pH 7.7, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 0.1 mM Na_3VO_4, 2 mM ditiothreitol, 20 μM ATP, and 5 μCi of [γ-^{32}P]ATP) for 20 min at 30 °C. For ERK assay, 50 μg of whole cell extract was immunoprecipitated with anti-ERK2 antibody, which exhibit cross-reactivity with ERK1 (Santa Cruz) for 2 h at 4 °C. Immunoprecipitates were then washed and incubated with 20 μl of kinase reaction buffer containing 1 μg/reaction of myelin basic protein (MBP, Sigma) as substrate. Reactions were stopped by adding 15 μl of SDS-loading buffer containing 10% β-mercaptoethanol. Phosphorylated GST-c-Jun and MBP proteins were eluted by boiling the samples for 5 min, and resolved on 10% and 15% SDS-polyacrylamide gel electrophoresis, respectively.

Transient Transfection Assays—Cells were seeded in six-well culture plates at 5×10^5 cells/well. A modified calcium phosphate precipitation procedure was used for transient transfection as described elsewhere (24). Briefly, 250 ng of reporter plasmid (−73Col-CAT) (25, 26) and 250 ng of β-galactosidase expression vector (pCH 110, Amersham Pharmacia Biotech) were mixed with carrier DNA (pBluescript) to 2.5 μg total of DNA/well. The day after transfection (18 h), cells were incubated in a medium containing 0.5% charcoal-treated FCS with *trans*-RA at the indicated concentrations and/or TPA (100 ng/ml) for an additional 24 h.

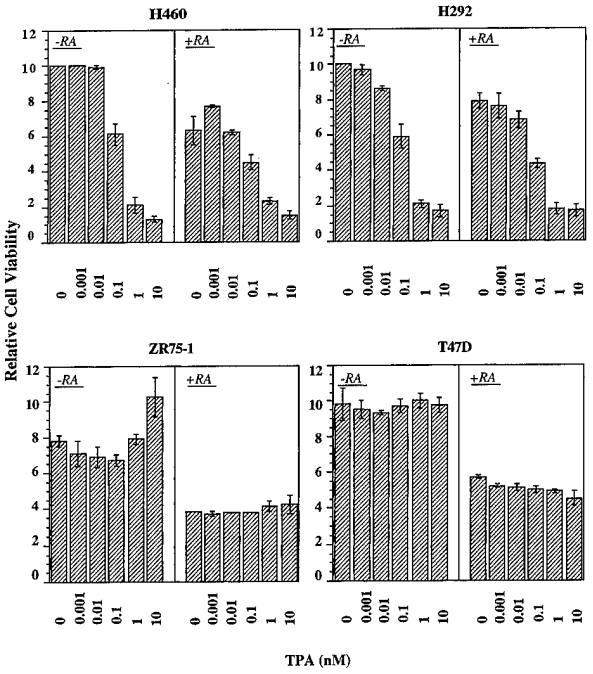

FIG. 1. **Growth inhibition by TPA and *trans*-RA in human lung cancer and breast cancer cell lines.** H460 and H292 lung cancer cells and ZR75-1 and T47D breast cancer cells were seeded at a cell density of 1,000 cells/well in 96-well plates. Cells were treated with 10^{-6} M *trans*-RA in the presence or absence of the indicated concentrations of TPA for 7 days, and viable cell number was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

RESULTS

Effect of TPA and trans-RA on the Growth of Lung and Breast Cancer Cells—We investigated the growth inhibitory effect of TPA and *trans*-RA on a number of human cancer cell lines, including lung cancer cell lines H460 and H292 and breast cancer cell lines ZR75-1 and T47D. As shown in Fig. 1, TPA inhibited the growth of both lung cancer cell lines by 80% at 1 or 10 nM. TPA, however, did not exhibit any inhibitory effect on growth of ZR75-1 and T47D breast cancer cells over a broad range of concentrations from 0.001 to 10 nM. Interestingly, TPA at 10 nM, enhanced the growth of ZR75-1 cells but not T47D cells. These data demonstrate that the effect of TPA on the growth of cancer cells is cell type-dependent, consistent with previous observations (27–30). In contrast to TPA, *trans*-RA showed growth inhibition on all cell lines investigated. In lung cancer cells H460 and H292, the percentage of inhibition was about 40 and 20%, respectively, whereas in breast cancer cells ZR75-1 and T47D *trans*-RA showed 60 and 50% inhibition, respectively (Fig. 1). When *trans*-RA was used in combination with TPA, an additive growth inhibitory effect was observed in H460 and H292 cells when low concentrations of TPA (0.01 nM and 0.1 nM) were used. Interestingly, the

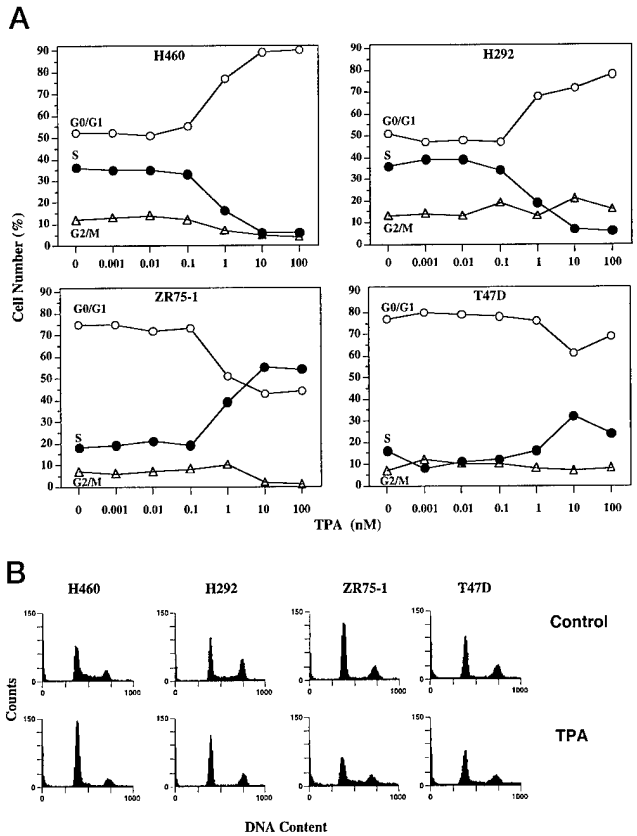

FIG. 2. **Effect of TPA on cell cycle progression.** *A*, effect of TPA concentrations on cell cycle progression of lung (H460, H292) and breast (ZR75-1, T47D) cancer cell lines. Cells were treated with the indicated concentrations of TPA for 15 h, stained with propidium iodide, and analyzed by flow cytometry. B, DNA content analysis by flow cytometry. Cells were treated with 10 nM TPA for 15 h, stained with propidium iodide, and analyzed by flow cytometry. The DNA content is presented as relative fluorescence. Cells in G_0/G_1 phase represent the first peak, and cells in the G_2/M phase represent the second peak. Cells in S phase are in the area between the G_0/G_1 and G_2/M phase peaks. Quantitation of cell cycle distribution is presented in Table I.

growth stimulatory effect of TPA on ZR75-1 cells was completely abolished by *trans*-RA (Fig. 1). Thus, *trans*-RA could enhance TPA-induced growth inhibition, but antagonizes TPA-induced cell proliferation.

Regulation of Cell Cycle Progression by TPA and trans-RA—To determine how TPA regulates growth of H460, H292, ZR75-1, and T47D cells, we investigated their cell cycle progression in response to TPA. The DNA content analysis showed that H460 and H292 cells underwent a stable G_0/G_1 arrest following 15 h of TPA treatment (Fig. 2, *A* and *B*). The entry of these cells into S phase was suppressed, while G_0/G_1 population was increased from 52 to 75%, and from 51% to 72%, respectively (Fig. 2, *A* and *B*, Table I). This data suggests that the growth inhibitory effect of TPA on these lung cancer cells is mainly due to its effect on cell cycle progression. When ZR75-1 and T47D breast cancer cells were analyzed, we observed a decrease in G_0/G_1 cell population (Fig. 2, *A* and *B*). This decrease was more apparent in ZR75-1 cells, with a percentage of cells in the G_0/G_1 phase decreasing from 75 to 43% when they were treated with 10 nM TPA for 15 h (Fig. 2, *A* and *B*, Table I). At the same time an increase in S phase cell population of 37 and 16% was observed in ZR75-1 and T47D cells, respectively. When the effect of *trans*-RA on cell cycle progression was analyzed, *trans*-RA alone did not show significant changes of G_0/G_1 arrest of H460, H292, and ZR75-1 cells, except a slight increase (5%) observed in T47D cells (Table I). When *trans*-RA was used together with TPA, it slightly increased TPA-induced G_0/G_1 in H460 and H292 cells (Table I). However, the inhibitory effect of TPA on G_0/G_1 phase in ZR75-1 and T47D cells was largely blocked by *trans*-RA (Table I). In ZR75-1 cells, in the absence of *trans*-RA, TPA decreased G_0/G_1 phase from 75 to 43%, which was reverted to 55% when *trans*-RA was present. The effect of TPA on G_0/G_1 phase in T47D cells was completely abolished by *trans*-RA. These data demonstrate that *trans*-RA could inhibit the effect of TPA on cell cycle progression of ZR75-1 and T47D cells.

Effect of TPA on Gene Expression—To obtain insight into the molecular mechanism by which TPA regulates cell cycle progression of cancer cells, we examined the effect of TPA on $p21^{WAF1}$, p53 and Cdk2 gene expression. When expression of $p21^{WAF1}$, an inhibitor of cyclin-dependent kinases (31, 32), was determined by Northern blot analysis, we observed that it was rapidly and strongly induced by TPA in H460, H292, ZR75-1, and T47D cells (Fig. 3, *A* and *B*). Interestingly, when expression of $p21^{WAF1}$ was analyzed by Western blotting, we found that it was only strongly induced in H292 and H460 cells (Fig. 4A). Slight induction of $p21^{WAF1}$ was observed in T47D cells, whereas ZR75-1 cells did not show any expression of $p21^{WAF1}$ either in the absence or in the presence of TPA (Fig. 4*B*). This observation suggests that $p21^{WAF1}$ expression is also regulated by a post-transcriptional regulatory mechanism. A 12-h treatment increased $p21^{WAF1}$ expression by about 8-fold in H460 cells and 12-fold in H292 cells (Fig. 4*A*). There was no evidence for p53 induction by TPA in these cell lines (Fig. 4*A*). The fact that $p21^{WAF1}$ was induced in H460 and H292 cells, in which p53 was not expressed or induced indicates that TPA-induced $p21^{WAF1}$ is p53-independent. We also examined the effect of TPA on expression of Cdk2 gene, which is also known to play a critical role in G_0/G_1 progression (13). The Cdk2 gene was highly expressed in all cell lines investigated (Fig. 4, *A* and *B*). However, TPA treatment for 12 h strongly inhibited expression of Cdk2 in H460 cells (Fig. 4*A*), while it had no effect in H292, ZR75-1, and T47D cells (Fig. 4, *A* and *B*). These data suggest that induction of G_0/G_1 arrest by TPA in H292 cells is likely due to its effect on $p21^{WAF1}$, whereas induction of $p21^{WAF1}$ and/or inhibition of Cdk2 expression may be responsible for TPA-induced G_0/G_1 arrest in H460 cells.

Effect of TPA on c-Jun and c-Fos Gene Expression—AP-1 is known to be associated with cell proliferation and it can be induced by TPA (1, 8). We then determined whether induction of AP-1 could account for enhancement of cell proliferation by TPA in ZR75-1 cells. As shown in Fig. 3*B*, expression of both c-Jun and c-Fos was strongly induced in ZR75-1 cells. Induction of c-Jun and c-Fos occurred as early as 30 min after TPA treatment. c-Jun was expressed in both H460 and H292 cells. However, its level of expression was not affected by TPA treatment. Expression of c-Fos in H460 and H292 cells was not influenced by TPA either (Fig. 3*A*). These data, therefore, suggest that induction of c-Jun and c-Fos may contribute to TPA-induced cell proliferation in ZR75-1 cells.

Induction of ERK by TPA Is Mainly Responsible for $p21^{WAF1}$ Induction—To study how TPA regulates $p21^{WAF1}$ expression and whether *trans*-RA modulates TPA activities in lung and breast cancer cell lines, we evaluated ERK activity that is known to regulate expression of $p21^{WAF1}$ (12). We examined the phosphorylation of MBP after immunoprecipitation of the whole cell extracts with anti-ERK2 antibody to determine ERK activity. As shown in Fig. 5, treatment of H460 cells with TPA for 30 min strongly induced ERK activity, while treatment with *trans*-RA did not show a clear effect on this activity. When *trans*-RA and TPA were used together TPA-induced ERK activity was not affected (Fig. 5). In ZR75-1 cells, ERK activity

TABLE I
Effect of TPA and RA on cell cycle distribution of human cancer cell lines

Cell cycle phase	Distribution in cell cycle[a]															
	H460				H292				ZR75–1				T47D			
	Control	TPA	RA	TPA + RA	Control	TPA	RA	TPA + RA	Control	TPA	RA	TPA + RA	Control	TPA	RA	TPA + RA
	%				%				%				%			
G_0/G_1	52	79	53	84	51	72	54	81	75	43	72	55	77	61	82	77
S	36	6	32	15	36	7	27	8	18	55	20	45	16	32	7	13
G_2/M	12	5	15	7	13	21	19	11	7	2	8	0	7	7	11	10

[a] Cell cycle distribution of cells treated with 10^{-6} M *trans*-RA and/or 10 nM TPA. Values represent the result of flow cytometry experiments. Representative histograms for cells treated with 10 nM TPA are shown in Fig. 2*B*.

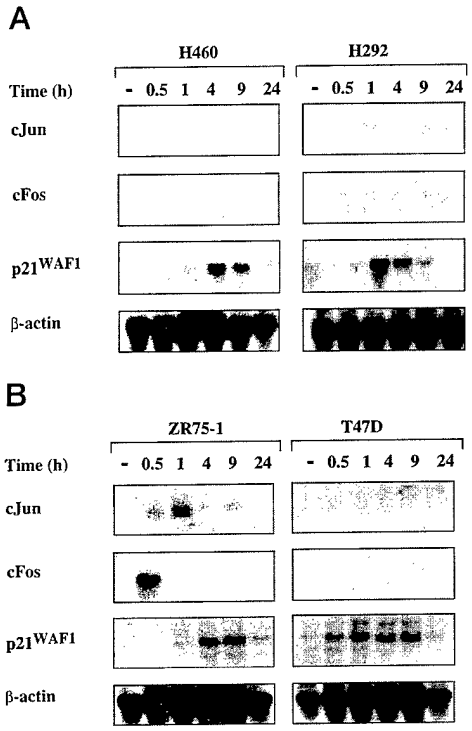

FIG. 3. **Regulation of c-Jun, c-Fos, and p21^{WAF1} expression by TPA.** *A*, lung cancer cell lines. *B*, breast cancer cell lines. Cells were treated with 10 nM TPA at the indicated times, and total RNAs were prepared and analyzed for the expression of the indicated genes by Northern blotting. Expression of β-actin is shown to ensure that equal amounts of RNAs were used, not treated with TPA.

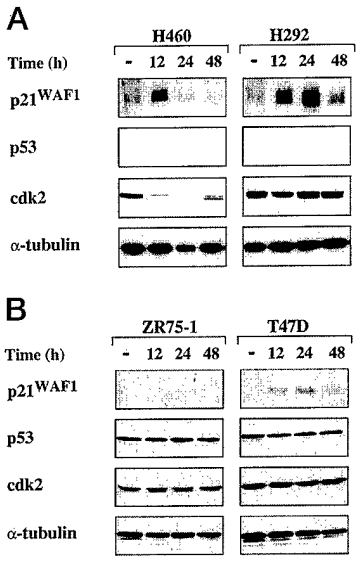

FIG. 4. **Analysis of TPA effect on p21^{WAF1}, p53, and Cdk2 expression by Western blot.** *A*, lung cancer cells. *B*, breast cancer cells. Cell extracts were prepared from the indicated cell lines treated with 10 nM TPA at the indicated times and analyzed for the expression of p21^{WAF1}, p53, and Cdk2. Expression of α-tubulin gene is shown as a control for protein loading. −, not treated with TPA.

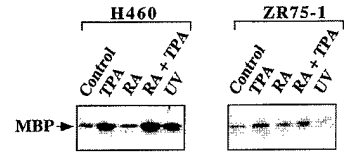

FIG. 5. **Effect of TPA and *trans*-RA on ERK activity.** ERK activity was determined in H460 lung and ZR75-1 breast cancer cells. Cells maintained in 0.5% FCS were treated for 30 min with TPA (10 nM) or UV (100 J/m^2). *trans-RA* was used at 10^{-6} M. When *trans*-RA was used in combination with TPA, cells were first incubated for 24 h with *trans*-RA before TPA addition. Whole cell extracts were prepared as described under "Experimental Procedures." Kinase activity was measured via the phosphorylation of the MBP protein. *Control*, untreated cells.

was slightly induced by TPA. Again, *trans*-RA did not show any effect on TPA-induced ERK activity in these cells. To determine whether induction of ERK activity by TPA is responsible for p21^{WAF1} induction, we examined the effect of PD98059, a specific inhibitor of Raf/ERK pathway, on p21^{WAF1} expression in H460 and ZR75-1 cells (Fig. 6). PD98059 alone (50 μM) did not show any effect on the expression of p21^{WAF1}. However, when PD98059 was used together with 10 nM TPA, induction of p21^{WAF1} by TPA was completely inhibited in both cell lines (Fig. 6). This suggests that induction of ERK is mainly responsible for p21^{WAF1} induction. Treatment of H460 cells with *trans*-RA for 24 h did not show a clear effect on p21^{WAF1} expression, consistent with the observation that *trans*-RA could not affect TPA-induced ERK activity. Thus, *trans*-RA has no effect on TPA-induced ERK/p21^{WAF1} pathway.

Induction of c-Jun and JNK by TPA Modulates Its Effect on the Growth of Cancer Cells—The above data demonstrate that TPA was able to induce c-Jun and c-Fos expression in ZR75-1 cells (Fig. 3*B*), suggesting that signaling that leads to AP-1 induction is functional in these cells. Transcriptional regulation of c-Jun expression is mainly mediated by a TPA-response element in its promoter, which binds to c-Jun/ATF-2 heterodimer (33). ATF-2 and c-Jun are activated mainly by JNK. The fact that c-Jun expression was rapidly induced by TPA in ZR75-1 cells (Fig. 3*B*) suggests that TPA may induce JNK in this cell line. To investigate this possibility, we analyzed JNK activation in H460 and ZR75-1 cells. JNK activity was determined by examination of the phosphorylation of GST-c-Jun in whole cell extracts prepared from H460 and ZR75-1 cells treated with different agents. As shown in Fig. 7, treatment of ZR75-1 cells with 10 nM TPA for 30 min strongly induced JNK activity in these cells. However, the same treatment failed to activate JNK in H460 cells. As a control, UV stimulation exhibited a strong activation of JNK in both cell lines. These data suggest that induction of c-Jun by TPA in ZR75-1 cells is likely due to activation of JNK. *trans*-RA has been shown to inhibit

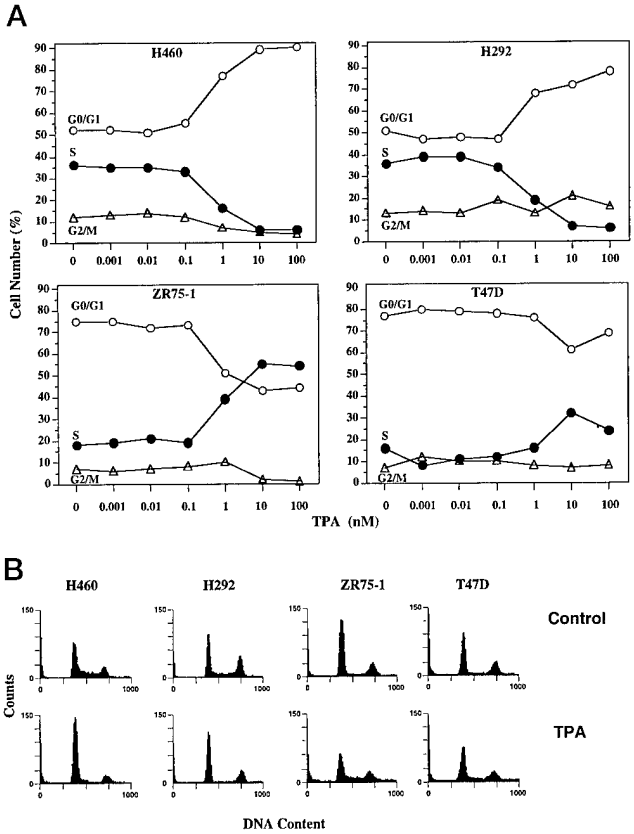

FIG. 2. **Effect of TPA on cell cycle progression.** *A*, effect of TPA concentrations on cell cycle progression of lung (H460, H292) and breast (ZR75-1, T47D) cancer cell lines. Cells were treated with the indicated concentrations of TPA for 15 h, stained with propidium iodide, and analyzed by flow cytometry. B, DNA content analysis by flow cytometry. Cells were treated with 10 nM TPA for 15 h, stained with propidium iodide, and analyzed by flow cytometry. The DNA content is presented as relative fluorescence. Cells in G_0/G_1 phase represent the first peak, and cells in the G_2/M phase represent the second peak. Cells in S phase are in the area between the G_0/G_1 and G_2/M phase peaks. Quantitation of cell cycle distribution is presented in Table I.

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Cell cycle phase	Distribution in cell cycle[a]															
	H460				H292				ZR75–1				T47D			
	Control	TPA	RA	TPA + RA	Control	TPA	RA	TPA + RA	Control	TPA	RA	TPA + RA	Control	TPA	RA	TPA + RA
	%				%				%				%			
G_0/G_1	52	79	53	84	51	72	54	81	75	43	72	55	77	61	82	77
S	36	6	32	15	36	7	27	8	18	55	20	45	16	32	7	13
G_2/M	12	5	15	7	13	21	19	11	7	2	8	0	7	7	11	10

[a] Cell cycle distribution of cells treated with 10^{-6} M *trans*-RA and/or 10 nM TPA. Values represent the result of flow cytometry experiments. Representative histograms for cells treated with 10 nM TPA are shown in Fig. 2*B*.

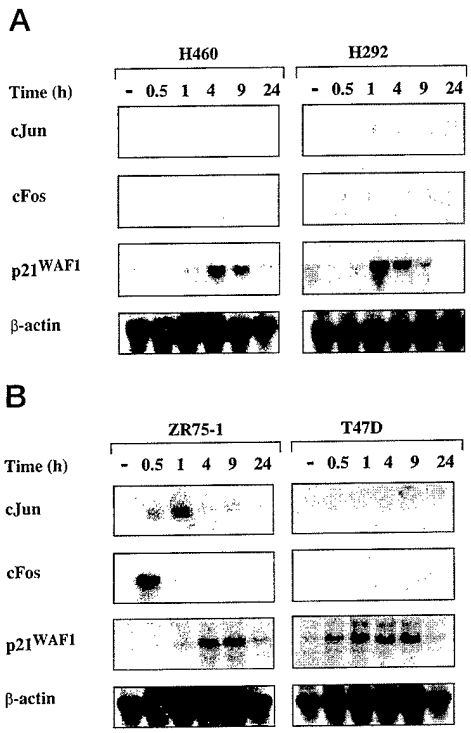

FIG. 3. **Regulation of c-Jun, c-Fos, and p21^{WAF1} expression by TPA.** *A*, lung cancer cell lines. *B*, breast cancer cell lines. Cells were treated with 10 nM TPA at the indicated times, and total RNAs were prepared and analyzed for the expression of the indicated genes by Northern blotting. Expression of β-actin is shown to ensure that equal amounts of RNAs were used, not treated with TPA.

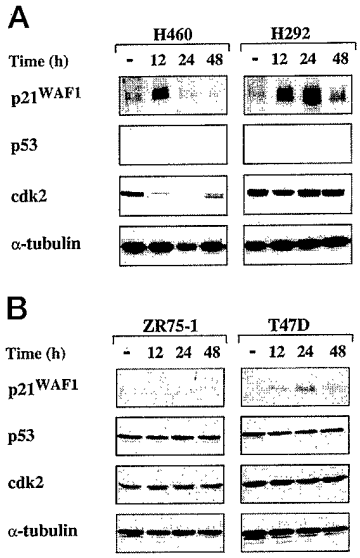

FIG. 4. **Analysis of TPA effect on p21^{WAF1}, p53, and Cdk2 expression by Western blot.** *A*, lung cancer cells. *B*, breast cancer cells. Cell extracts were prepared from the indicated cell lines treated with 10 nM TPA at the indicated times and analyzed for the expression of p21^{WAF1}, p53, and Cdk2. Expression of α-tubulin gene is shown as a control for protein loading. −, not treated with TPA.

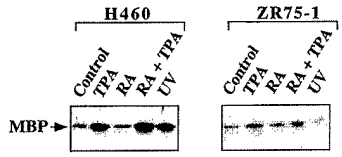

FIG. 5. **Effect of TPA and *trans*-RA on ERK activity.** ERK activity was determined in H460 lung and ZR75-1 breast cancer cells. Cells maintained in 0.5% FCS were treated for 30 min with TPA (10 nM) or UV (100 J/m^2). *trans-RA* was used at 10^{-6} M. When *trans*-RA was used in combination with TPA, cells were first incubated for 24 h with *trans*-RA before TPA addition. Whole cell extracts were prepared as described under "Experimental Procedures." Kinase activity was measured via the phosphorylation of the MBP protein. *Control*, untreated cells.

was slightly induced by TPA. Again, *trans*-RA did not show any effect on TPA-induced ERK activity in these cells. To determine whether induction of ERK activity by TPA is responsible for p21^{WAF1} induction, we examined the effect of PD98059, a specific inhibitor of Raf/ERK pathway, on p21^{WAF1} expression in H460 and ZR75-1 cells (Fig. 6). PD98059 alone (50 μM) did not show any effect on the expression of p21^{WAF1}. However, when PD98059 was used together with 10 nM TPA, induction of p21^{WAF1} by TPA was completely inhibited in both cell lines (Fig. 6). This suggests that induction of ERK is mainly responsible for p21^{WAF1} induction. Treatment of H460 cells with *trans*-RA for 24 h did not show a clear effect on p21^{WAF1} expression, consistent with the observation that *trans*-RA could not affect TPA-induced ERK activity. Thus, *trans*-RA has no effect on TPA-induced ERK/p21^{WAF1} pathway.

Induction of c-Jun and JNK by TPA Modulates Its Effect on the Growth of Cancer Cells—The above data demonstrate that TPA was able to induce c-Jun and c-Fos expression in ZR75-1 cells (Fig. 3*B*), suggesting that signaling that leads to AP-1 induction is functional in these cells. Transcriptional regulation of c-Jun expression is mainly mediated by a TPA-response element in its promoter, which binds to c-Jun/ATF-2 heterodimer (33). ATF-2 and c-Jun are activated mainly by JNK. The fact that c-Jun expression was rapidly induced by TPA in ZR75-1 cells (Fig. 3*B*) suggests that TPA may induce JNK in this cell line. To investigate this possibility, we analyzed JNK activation in H460 and ZR75-1 cells. JNK activity was determined by examination of the phosphorylation of GST-c-Jun in whole cell extracts prepared from H460 and ZR75-1 cells treated with different agents. As shown in Fig. 7, treatment of ZR75-1 cells with 10 nM TPA for 30 min strongly induced JNK activity in these cells. However, the same treatment failed to activate JNK in H460 cells. As a control, UV stimulation exhibited a strong activation of JNK in both cell lines. These data suggest that induction of c-Jun by TPA in ZR75-1 cells is likely due to activation of JNK. *trans*-RA has been shown to inhibit

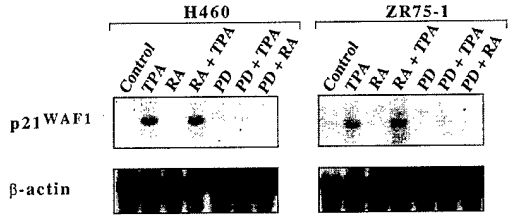

FIG. 6. **PD98059 inhibits TPA-induced p21^{WAF1}.** Cells maintained in 0.5% FCS were first treated with 10^{-6} M *trans*-RA for 24 h. One day later PD98059 (*PD*) (50 μM) was added, and the cells were incubated for 4 h before addition of TPA (10 nM). Cells were then incubated for an additional 15 h. Total RNAs were prepared from H460 and ZR75-1 cells and analyzed for the expression of p21^{WAF1} by Northern blot. Expression of β-actin is shown to ensure that equal amounts of RNAs were used. *Control*, untreated cells.

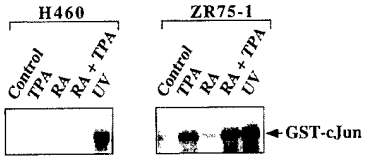

FIG. 7. **Effect of TPA and *trans*-RA on JNK activity.** JNK activity was determined in H460 and ZR75-1 breast cancer cells. Cells were treated as described in the legend to Fig. 5. Kinase activity was measured via the phosphorylation of GST-c-Jun protein.

JNK activity in various cell types (34). To study whether inhibition of TPA-induced cell proliferation in ZR75-1 cells by *trans*-RA was due to inhibition of JNK activity, we cotreated cells with TPA and *trans*-RA. *Trans*-RA alone did not show any effect on JNK activity in ZR75-1 cells (Fig. 7). It also failed to inhibit TPA-induced JNK activity. Thus, it is unlikely that *trans*-RA antagonizes TPA effect by inhibiting TPA-induced JNK activity in ZR75-1 cells.

Inihibtion of AP-1 Activity by trans-RA—*trans*-RA can antagonize TPA effect through inhibition of AP-1 activity (20). We then investigated the effect of *trans*-RA on transactivation of collagenase promoter which contains an AP-1 binding site (25, 26). When reporter construct containing the collagenase promoter linked with the CAT gene, −73Col-CAT, was transiently transfected into ZR75-1 cells, treatment of the cells with TPA strongly induced the reporter transcription (Fig. 8*A*). In contrast, the −73-Col-CAT reporter was highly active in H292 cells, and addition of TPA did not significantly induce transcription of the reporter gene (Fig. 8*B*). This suggests that TPA could strongly induce AP-1 activity in ZR75-1 cells but not in H292 cells, consistent with regulation of c-Jun and c-Fos expression by TPA (Fig. 3, *A* and *B*). In ZR75-1 and cells, *trans*-RA alone did not show any effect on transcription of the collagenase promoter (data not shown). However, when it was used together with TPA, TPA-induced reporter gene transcription was impaired in a *trans*-RA concentration dependent manner (Fig. 8*A*). *trans*-RA, at 10^{-6} M, almost completely abolished TPA-induced reporter activity. Thus *trans*-RA could repress TPA-induced AP-1 activity, which may contribute to its inhibitory effect on the growth-stimulatory activity of TPA in ZR75-1 cells. However, in H292 cells, *trans*-RA significantly repressed the basal reporter activity. A similar degree of inhibition was also observed in the presence of TPA (Fig. 8*B*). This suggests that inhibition of the constitutive AP-1 activity by *trans*-RA may be the mechanism by which *trans*-RA inhibits proliferation of H460 and H292 cells and may explain the additive growth inhibitory effect by *trans*-RA and TPA combination on these cells.

DISCUSSION

In this study, we investigated the effect of two potent growth regulators, TPA and *trans*-RA, on the growth of lung (H460, H292) and breast (ZR75-1, T47D) cancer cell lines and their interaction. Our results demonstrate that TPA exerts either inhibition or stimulation of cancer cell proliferation, whereas *trans*-RA shows various degrees of growth inhibition in all cell lines investigated (Fig. 1). When *trans*-RA was used together with TPA an additive growth inhibitory effect was observed in H460 and H292 cells, whereas it completely abolished the growth-stimulatory effect of TPA on ZR75-1 breast cancer cells. We also show that induction of G_0/G_1 arrest by TPA in H460 and H292 cells is accompanied by induction of p21^{WAF1} due to activation of ERK pathway (Fig. 5) and/or inhibition of Cdk2 gene expression, whereas stimulation of cell proliferation in ZR75-1 cells by TPA is associated with induction of c-Jun and c-Fos expression and JNK activation (Fig. 3*B*, Fig. 7). Although *trans*-RA did not interfere with TPA-induced p21^{WAF1} expression and ERK activity, it strongly inhibited TPA-induced AP-1 activity without affecting TPA-induced JNK activity (Figs. 6, 7, and 8).

The growth inhibitory effect of TPA on H460 and H292 cells is mainly due to arrest of these cells in G_0/G_1 phase (Fig. 2, *A* and *B*, Table I), due to induction of p21^{WAF1} expression in H292 cells and/or inhibition of Cdk2 expression in H460 cells (Figs. 3 and 4). P21^{WAF1} can interact with cyclin-Cdk complexes and is capable of inhibiting kinase activities associated with these complexes (31, 32). A major target of p21^{WAF1} inhibition is the cyclin-Cdk2 kinase complex whose activity is required for G_0/G_1 progression into S phase (13, 14). TPA has been shown to induce p21^{WAF1} in a variety of cell types (35, 36). Inhibition of Cdk2 expression by TPA has also been observed during the differentiation of HL60 leukemia cells (37). Thus, the increase of p21^{WAF1} in H292 cells and/or the decrease of Cdk2 expression in H460 cells upon TPA treatment may be sufficient to inhibit kinase activity required for G_0/G_1 progression into S phase. These results, taken together with previous findings (35–37), suggest that induction of p21^{WAF1} and/or inhibition of Cdk2 expression may play a causative role in TPA-induced growth arrest. Interestingly, p21^{WAF1} messenger was also highly induced by TPA in ZR75-1 cells (Fig. 3*B*). However, we did not detect any p21^{WAF1} protein product (Fig. 4*B*). A previous study (38) demonstrated that expression of p21^{WAF1} often involves a post-transcriptional mechanism. The inability of ZR75-1 cells to express p21^{WAF1} protein product suggests that p21^{WAF1} transcript may be unstable in these cells. Although p21^{WAF1} was also induced in T47D cells (Figs. 3 and 4), the degree of induction is much less than those observed in H460 and H292 cells, and may not be enough to confer a G_0/G_1 arrest by TPA in the cells (Fig. 2 and Table I). Induction of p21^{WAF1} by DNA damage requires p53 (39). However, under many experimental conditions p21^{WAF1} can be induced through p53-independent pathways (35–37). Our observation that p21^{WAF1} was induced by TPA in H460 and H292 cells without affecting p53 expression (Figs. 3*A* and 4*A*) suggests that TPA induced p21^{WAF1} expression is p53-independent. In studying possible signaling pathway leading to p21^{WAF1} induction in H460 cells, we found that TPA strongly activated ERK kinase activity, while it had no effect on JNK activity (Figs. 5 and 7). Our observation that PD98059, a specific inhibitor of Raf/ERK pathway, abolished p21^{WAF1} induction by TPA (Fig. 6) suggests that ERK pathway is responsible for TPA-induced p21^{WAF1} in both H460 and ZR75-1 cells. A similar study also showed that induction of p21^{WAF1} by TPA in SKBR3 breast cancer and LNCaP prostate cancer cells is attributed to stimulation of Raf-1/MEK pathway (40).

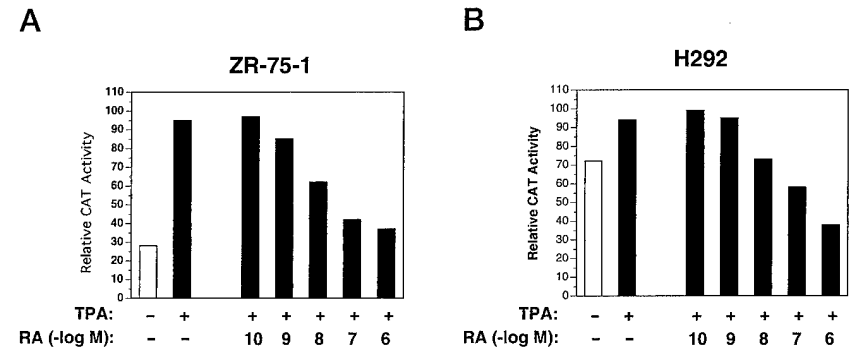

FIG. 8. **Inhibition of AP-1 activity by *trans*-RA.** *A*, inhibition of TPA-induced AP-1 activity by *trans*-RA in ZR75-1 breast cancer cells. *B*, inhibition of AP-1 activity by *trans*-RA in H292 lung cancer cells. The −73Col-CAT reporter was transfected into the cells. After transfection, cells were incubated in medium containing 0.5% FCS and treated with the indicated concentrations of *trans*-RA and/or TPA (100 ng/ml). 24 h later, the cells were harvested, and CAT activities were determined.

Our results also demonstrate that TPA can stimulate proliferation of ZR75-1 breast cancer cells (Fig. 1). Although TPA acts as a potent inducer of growth arrest of many cancer cell types, the mitogenic effect of TPA has been also described (5, 7). TPA inhibited the growth of malignant melanoma cells, while the growth of normal melanocytes was stimulated (41). Similarly, two NIH3T3 clones, N3T3 and P-3T3, showed opposite response to TPA (42); TPA inhibited the growth of N-3T3 cells, while it stimulated the growth of P-3T3 cells. Our present study further demonstrates the diverse functions of TPA, which is likely determined by the cell context, which in turn dictates the biological outcome of TPA. The molecular mechanism by which TPA induces growth arrest is well studied. However, how TPA stimulates cell proliferation is less understood. When we investigated the effect of TPA in ZR75-1 cells, we found that it strongly induced expression of c-Jun and c-Fos (Fig. 3*B*). Such an effect was not seen in H460 cells (Fig. 3*A*). C-jun and c-Fos, the components of AP-1, act as transcriptional factors for numerous genes, and overexpression of these genes is often associated with cell proliferation and malignant transformation (8). Thus, induction of AP-1 activity by TPA may contribute to its growth stimulatory effect in ZR75-1 cells. Our results also demonstrate that induction of c-Jun and c-Fos expression by TPA is likely due to activation of JNK, which is known to activate c-Jun promoter through phosphorylation of c-Jun and ATF2 that bind to a TPA-response element in the c-Jun promoter as heterodimer (33). Interestingly, activation of JNK was not observed in H460 cells (Fig. 7), which could explain the inability of TPA to induce c-Jun expression in these cells. Taken together, our results demonstrate that the pleiotropic effects of TPA are mediated by multiple signaling pathways whose operation is largely determined by the cellular context.

Previous studies have demonstrated that *trans*-RA could effectively counteract TPA effects (20, 25, 26, 43). *trans*-RA could prevent transformation of JB6 mouse epidermal cells promoted by TPA (43) and counteract the effect of TPA on expression of fibronectin gene in fibroblasts (44), transglutaminase 1 gene in keratinocytes (45), as well as collagenase (46), stromelysin (47), and ornithine decarboxylase (48). In this study, we found that *trans*-RA exerted different effects on TPA activities in different cell lines. In H460 and H292 cells, pretreatment of the cells with *trans*-RA increased the growth inhibitory effect of TPA. An additive effect was observed when 1 nM or less TPA was used. In contrast, *trans*-RA antagonized the growth stimulatory effect of TPA on ZR75-1 cells. In the absence of *trans*-RA, TPA enhanced proliferation of ZR75-1 cells, which was almost completely abolished when *trans*-RA was added (Fig. 1). Although $p21^{WAF1}$ promoter contains a RA response element (49) we did not observe any effect of *trans*-RA on $p21^{WAF1}$ expression (Fig. 6). This suggests that the growth inhibitory effect of *trans*-RA is unlikely due to induction of $p21^{WAF1}$. In studying the antagonism effect of *trans*-RA on TPA activity in ZR75-1 cells, we found that *trans*-RA could effectively inhibit TPA-induced AP-1 activity (Fig. 8*A*). TPA could induce endogenous AP-1 activity as demonstrated by our observation that TPA strongly induced collagenase promoter activity in ZR75-1 cells (Fig. 8*A*). The TPA-induced AP-1 activity in ZR75-1 cells was largely inhibited by *trans*-RA. This is consistent with previous observations showing that *trans*-RA could antagonize AP-1 activity in HeLa cells (25) and suggests that *trans*-RA may antagonize the growth-stimulatory effect of TPA in ZR75-1 cells through repression of TPA-induced AP-1 activity. Interestingly, *trans*-RA could also inhibit the constitutive AP-1 activity in H292 cells (Fig. 8*B*), suggesting that the growth inhibitory effect of *trans*-RA in these cells may be in part due to inhibition of AP-1 activity which may contribute to the additive growth inhibitory effect of TPA and *trans*-RA combinatory treatment observed in these cells (Fig. 1). A previous study demonstrated that *trans*-RA inhibited JNK activity in HeLa cells (34). However, we did not detect any effect of *trans*-RA on TPA-induced JNK activity in ZR75-1 cells (Fig. 7). Thus a mechanism other than inhibition of JNK activity may be responsible for inhibition of AP-1 activity by *trans*-RA.

Together, our results demonstrate that TPA could exert mitogenic or anti-mitogenic effect through different signaling transduction pathways in a cell type specific manner. *trans*-RA may enhance anti-mitogenic effect of TPA and antagonize its mitogenic effect through inhibition of AP-1 activity. These two potent regulators of cell growth, through their interaction, are expected to play a critical role in the regulation of cancer cell growth.

Acknowledgment—We thank Paula Kovack for preparation of the manuscript.

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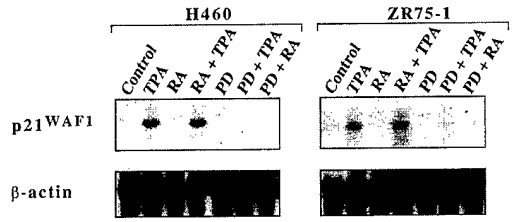

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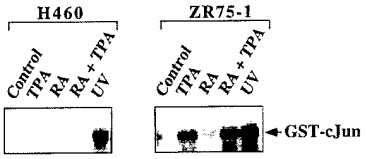

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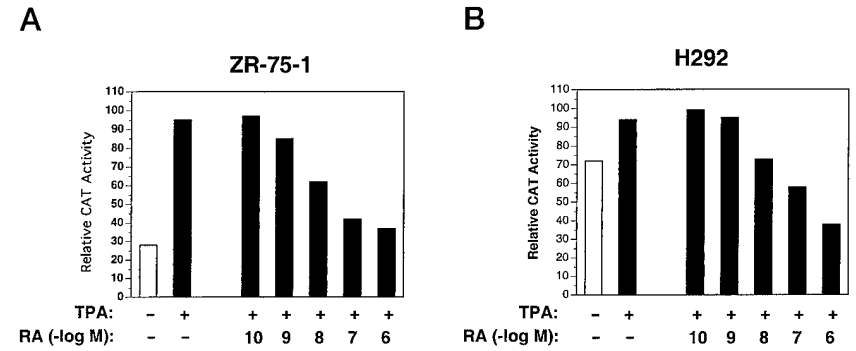

FIG. 8. **Inhibition of AP-1 activity by *trans*-RA.** *A*, inhibition of TPA-induced AP-1 activity by *trans*-RA in ZR75-1 breast cancer cells. *B*, inhibition of AP-1 activity by *trans*-RA in H292 lung cancer cells. The −73Col-CAT reporter was transfected into the cells. After transfection, cells were incubated in medium containing 0.5% FCS and treated with the indicated concentrations of *trans*-RA and/or TPA (100 ng/ml). 24 h later, the cells were harvested, and CAT activities were determined.

Our results also demonstrate that TPA can stimulate proliferation of ZR75-1 breast cancer cells (Fig. 1). Although TPA acts as a potent inducer of growth arrest of many cancer cell types, the mitogenic effect of TPA has been also described (5, 7). TPA inhibited the growth of malignant melanoma cells, while the growth of normal melanocytes was stimulated (41). Similarly, two NIH3T3 clones, N3T3 and P-3T3, showed opposite response to TPA (42); TPA inhibited the growth of N-3T3 cells, while it stimulated the growth of P-3T3 cells. Our present study further demonstrates the diverse functions of TPA, which is likely determined by the cell context, which in turn dictates the biological outcome of TPA. The molecular mechanism by which TPA induces growth arrest is well studied. However, how TPA stimulates cell proliferation is less understood. When we investigated the effect of TPA in ZR75-1 cells, we found that it strongly induced expression of c-Jun and c-Fos (Fig. 3*B*). Such an effect was not seen in H460 cells (Fig. 3*A*). C-jun and c-Fos, the components of AP-1, act as transcriptional factors for numerous genes, and overexpression of these genes is often associated with cell proliferation and malignant transformation (8). Thus, induction of AP-1 activity by TPA may contribute to its growth stimulatory effect in ZR75-1 cells. Our results also demonstrate that induction of c-Jun and c-Fos expression by TPA is likely due to activation of JNK, which is known to activate c-Jun promoter through phosphorylation of c-Jun and ATF2 that bind to a TPA-response element in the c-Jun promoter as heterodimer (33). Interestingly, activation of JNK was not observed in H460 cells (Fig. 7), which could explain the inability of TPA to induce c-Jun expression in these cells. Taken together, our results demonstrate that the pleiotropic effects of TPA are mediated by multiple signaling pathways whose operation is largely determined by the cellular context.

Previous studies have demonstrated that *trans*-RA could effectively counteract TPA effects (20, 25, 26, 43). *trans*-RA could prevent transformation of JB6 mouse epidermal cells promoted by TPA (43) and counteract the effect of TPA on expression of fibronectin gene in fibroblasts (44), transglutaminase 1 gene in keratinocytes (45), as well as collagenase (46), stromelysin (47), and ornithine decarboxylase (48). In this study, we found that *trans*-RA exerted different effects on TPA activities in different cell lines. In H460 and H292 cells, pretreatment of the cells with *trans*-RA increased the growth inhibitory effect of TPA. An additive effect was observed when 1 nM or less TPA was used. In contrast, *trans*-RA antagonized the growth stimulatory effect of TPA on ZR75-1 cells. In the absence of *trans*-RA, TPA enhanced proliferation of ZR75-1 cells, which was almost completely abolished when *trans*-RA was added (Fig. 1). Although $p21^{WAF1}$ promoter contains a RA response element (49) we did not observe any effect of *trans*-RA on $p21^{WAF1}$ expression (Fig. 6). This suggests that the growth inhibitory effect of *trans*-RA is unlikely due to induction of $p21^{WAF1}$. In studying the antagonism effect of *trans*-RA on TPA activity in ZR75-1 cells, we found that *trans*-RA could effectively inhibit TPA-induced AP-1 activity (Fig. 8*A*). TPA could induce endogenous AP-1 activity as demonstrated by our observation that TPA strongly induced collagenase promoter activity in ZR75-1 cells (Fig. 8*A*). The TPA-induced AP-1 activity in ZR75-1 cells was largely inhibited by *trans*-RA. This is consistent with previous observations showing that *trans*-RA could antagonize AP-1 activity in HeLa cells (25) and suggests that *trans*-RA may antagonize the growth-stimulatory effect of TPA in ZR75-1 cells through repression of TPA-induced AP-1 activity. Interestingly, *trans*-RA could also inhibit the constitutive AP-1 activity in H292 cells (Fig. 8*B*), suggesting that the growth inhibitory effect of *trans*-RA in these cells may be in part due to inhibition of AP-1 activity which may contribute to the additive growth inhibitory effect of TPA and *trans*-RA combinatory treatment observed in these cells (Fig. 1). A previous study demonstrated that *trans*-RA inhibited JNK activity in HeLa cells (34). However, we did not detect any effect of *trans*-RA on TPA-induced JNK activity in ZR75-1 cells (Fig. 7). Thus a mechanism other than inhibition of JNK activity may be responsible for inhibition of AP-1 activity by *trans*-RA.

Together, our results demonstrate that TPA could exert mitogenic or anti-mitogenic effect through different signaling transduction pathways in a cell type specific manner. *trans*-RA may enhance anti-mitogenic effect of TPA and antagonize its mitogenic effect through inhibition of AP-1 activity. These two potent regulators of cell growth, through their interaction, are expected to play a critical role in the regulation of cancer cell growth.

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Orphan Receptor COUP-TF Is Required for Induction of Retinoic Acid Receptor β, Growth Inhibition, and Apoptosis by Retinoic Acid in Cancer Cells

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Retinoic acid receptor β (RARβ) plays a critical role in mediating the anticancer effects of retinoids. Expression of RARβ is highly induced by retinoic acid (RA) through a RA response element (βRARE) that is activated by heterodimers of RARs and retinoid X receptors (RXRs). However, RARβ induction is often lost in cancer cells despite expression of RARs and RXRs. In this study, we provide evidence that orphan receptor COUP-TF is required for induction of RARβ expression, growth inhibition, and apoptosis by RA in cancer cells. Expression of COUP-TF correlates with RARβ induction in a variety of cancer cell lines. In addition, stable expression of COUP-TF in COUP-TF-negative cancer cells restores induction of RARβ expression, growth inhibition, and apoptosis by RA, whereas inhibition of COUP-TF by expression of COUP-TF antisense RNA represses the RA effects. In a transient transfection assay, COUP-TF strongly induced transcriptional activity of the RARβ promoter in a RA- and RARα-dependent manner. By mutation analysis, we demonstrate that the effect of COUP-TF requires its binding to a DR-8 element present in the RARβ promoter. The binding of COUP-TF to the DR-8 element synergistically increases the RA-dependent RARα transactivation function by enhancing the interaction of RARα with its coactivator CREB binding protein. These results demonstrate that COUP-TF, by serving as an accessory protein for RARα to induce RARβ expression, plays a critical role in regulating the anticancer activities of retinoids.

Retinoids, a class of natural and synthetic vitamin A analogs, exert profound effects on many biological processes, including cell proliferation and differentiation, vision, reproduction, morphogenesis, and pattern formation, both in normal and transformed cells (9, 30). They have been well recognized as promising agents for the prevention of human cancers (9, 14, 30, 58). Their therapeutic potential has also received a great amount of attention, since all-*trans*-retinoic acid (RA) showed dramatic antitumor effects in patients with acute promyelocytic leukemia (58). However, retinoid resistance associated with many different types of cancer has prevented the further application of retinoids (14, 58).

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors (RARs) and retinoid X receptors (RXRs) (19, 34, 67). 9-*cis*-RA is a high-affinity ligand for both RARs and RXRs, whereas all-*trans*-RA is a ligand for only RARs. RARs and RXRs are encoded by three distinct genes (α, β, and γ) and are members of the steroid/thyroid hormone receptor superfamily, which function as ligand-activated transcription factors (19, 34, 67). RARs and RXRs primarily function as RXR-RAR heterodimers that bind to a variety of RA response elements (RAREs) and regulate their transactivation function (19, 34, 67). Transcriptional activation by retinoid receptors requires a carboxy-terminal helical region, termed activation function-2, that forms part of the ligand-binding pocket and that undergoes a conformational change required for the recruitment of coactivator proteins, such as CREB binding protein (CBP) (18). This appears to provide a direct link to the core transcriptional machinery and modulates chromatin structure (62).

In addition to retinoid receptors, a number of orphan receptors whose ligands are unknown have been implicated in the regulation of the retinoid response (47). One of the factors is COUP-TF. COUP-TF is encoded by two distinct genes, COUP-TFI (ear-3) (36, 56) and COUP-TFII (ARP-1) (22). Both genes show an exceptionally high degree of homology, and their expression patterns often overlap, suggesting that they may serve redundant functions (47). However, each factor possesses its own distinct expression profile during development (47). A null mutation of COUP-TFI resulted in defects in neurogenesis, axon guidance, and arborization (46), whereas deletion of COUP-TFII resulted in striking defects in angiogenesis, vascular remodeling, and fetal heart development (41). COUP-TF was originally shown to stimulate gene transcription (40). However, subsequent work has demonstrated that COUP-TF can repress the transcription induced by a number of nuclear receptors including RARs, thyroid hormone receptors, and vitamin D receptor (20, 24, 53, 59). It has been recently reported that COUP-TFs can function as positive regulators for many different genes. In the arrestin gene promoter, a DR-7 element mediates the positive transcriptional effect of COUP-TF (32), while in the other genes, such as the trout estrogen receptor (23), the phosphoenolpyruvate carboxykinase (10), the vHNF1 (45), the human immunodeficiency virus long terminal repeat (49), and the NGFI-A (44) genes, the positive-transcription function of COUP-TF is mediated through its interaction with other transcriptional factors. For the NGFI-A gene, COUP-TF enhances transcription by recruiting coactivator SRC-1 through its interaction with SP-1 (44).

Retinoids exert their anticancer activities mainly through their abilities to modulate the growth, differentiation, and ap-

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optosis of cancer cells. Recent studies have indicated that RARβ plays a critical role in mediating the growth-inhibitory effect of retinoids in many different types of cancer cells, including those of breast cancer (27, 29), lung cancer (28), ovarian cancer (42), prostate cancer (2), neuroblastoma (6), renal cell carcinoma (12), pancreatic cancer (17), liver cancer (26), and head and neck cancer (●●●). Expression of RARβ in RARβ-negative cancer cells restored RA-induced growth inhibition and apoptosis, whereas inhibition of RARβ expression in RARβ-positive cancer cells abolished RA effects (27–29). In addition, transgenic mice expressing RARβ antisense sequences showed increased incidence of lung tumor (1), whereas suppression of RARβ expression was responsible for diminished anticancer activities of retinoids in animals (57). Moreover, up-regulation of RARβ is associated with a positive clinical response to retinoids in patients with premalignant oral lesions (31, 50).

The finding that a deletion of the short arm of chromosome 3p24, close to where the RARβ gene maps, occurs with high frequency in human tumors (21, 38) led to studies on abnormalities of the RARβ gene and its expression. These studies revealed a high frequency of abnormal expression of the RARβ gene, but not of genes of the other RAR subtypes, in human cancer cell lines and primary human cancer tissues (8, 15, 16, 39, 55, 68, 69). Thus, the loss of RARβ may be a key mechanism by which cancer cells escape normal growth control and a contributing factor in cancer development (35, 63). Indeed, it has been shown that loss of RARβ is an early event in carcinogenesis (43, 60, 63, 64) and may be involved in liver cancer development (4).

How RARβ expression is regulated and how its expression is lost in cancer cells remain largely unknown and are subjects of intensive study. Expression of RARβ is highly induced by RA through a RARE (βRARE) present in its promoter (5, 13, 51) that is activated by RAR-RXR heterodimers (54, 65). However, we and others have recently demonstrated that expression of RARs and RXRs is not sufficient to render RARβ expression responsive to RA (37, 55, 68, 70). In the majority of lung cancer cell lines, RARβ expression could not be induced by RA, despite expression of RARs and RXRs (68). Similarly, retinoid refractoriness occurs during lung carcinogenesis despite the expression of functional retinoid receptors (70). Thus, factors other than RARs and RXRs are required for RA to induce RARβ expression.

We have previously demonstrated that expression of COUP-TF is positively correlated with RARβ induction and growth inhibition by RA in lung cancer cell lines (61). In this study, we further investigated the effect of COUP-TF in the regulation of RA-dependent RARβ induction and the underlying molecular mechanism. Our results demonstrate that COUP-TF is required for RA to induce RARβ expression, growth inhibition, and apoptosis in cancer cells. In addition, our studies showed that COUP-TF could strongly induce transcriptional activity of the RARβ promoter in a RA- and RARα-dependent manner through its binding to a DR-8 element present in the promoter. Furthermore, we observed that COUP-TF, through its interaction with RARα, strongly enhanced the interaction of RARα with its coactivator CBP, demonstrating that COUP-TF induces RARβ promoter transcription by acting as an accessory protein for RARα to recruit its coactivator.

MATERIALS AND METHODS

Cell culture. CV-1, HeLa, MDA-MB231, and MDA-MB468 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS). Calu-6, HT-1376, J82, SK-MES-1, and 5637 cells were maintained in minimal essential medium supplemented with 10% FCS. H292, H520, H460, H596, H441, and H661 cells were grown in RPMI 1640 supplemented with 10% FCS. A-549 cells were maintained in F12 medium supplemented with 10% FCS.

Plasmid constructions. The RARβ promoter reporter (−745RARβCAT) has been described previously (11). The coding sequence of RARα was inserted into the multiple cloning sites of the eukaryotic expression vector pECE (65). The insertion of COUP-TFI cDNA into pRC/CMV vector (Invitrogen, San Diego, Calif.) in sense and antisense orientations followed the procedure described previously (29). The RARβ promoter deletion mutants were created by cloning *Bam*HI-flanked PCR products derived from −745RARβ promoter into the pBluescript chloramphenicol acetyltransferase (CAT) plasmid (11). The forward primers were as follows: for the −516RARβ promoter, CATGGATCCTAGCC ATTCTCGTTCTACAGT; for the −300RARβ promoter, CATGGATCCAGA AGTTGGTGCTCAACGTGA; for the −126RARβ promoter, CATGGATCC AGCTCTGTGAGAATCCTGGG. The oligonucleotide CATGGATCCTACCC CGACGGTGCCCAGA was used as the reverse primer for the above mutants. The −60RARβ promoter mutant was constructed by subcloning a *Sma*I/*Bam*HI-flanked fragment from the RARβ promoter into the pBluescript CAT plasmid. The COUP-TF-RE and βRARE mutant constructs were generated by ligating the mutated RARβ promoter PCR products into the pBluescript CAT vector. The following primers were used to incorporate the appropriate mutations: TCCCCCGGGCTGCTAACCTTCAAATGACCCAAGTGACATCACCAA for COUP-TF-RE/M1, TCCCCCGGGCTGCTAACCTTCAAATGACCCAACTA GTCGAGCATCACCAA for COUP-TF-RE/M2, TCCCCCGGGTAGAACTC ACCGAAAGTTCAC for βRARE/M1, and TCCCGGGTAGGGTTCACCGA GTTCAC for βRARE/M2. The COUP-TF-RE-tk-CAT reporter was obtained by inserting the DR-8 synthetic oligonucleotide into the *Bam*HI site of pBL-CAT2 (65). For COUP-TFII receptor mutations, pBSCOUP-TFII deletion mutants were obtained by cloning PCR products from COUP-TFII into pBS-COUP-TFII. The oligonucleotide CATCGAGTGCGTGAGACGGGAAG CGGTG was used as the forward primer, while the reverse primers were as follows: GCCATGTCGACTCAGTTAAAACTGCTGCC for pBS-COUP-TFII/Δ7, TGCTGGTCGACTATAACATATCCCGGATG for pBS-COUP-TFII/Δ14, and ACCTGTCGACTAGACGAAAAACAATTGC for pBS COUP-TFII/Δ30. pBS-COUP-TFII/Δ80 was obtained by digesting pBS-COUP-TFII with *Hin*dIII, whereas pBS-COUP-TFII/Δ108 was obtained by digesting pBS-COUP-TFII with *Hin*dII. pBS-COUP-TFII/Δ179 was generated by subcloning an *Eco*RI/*Pst*I COUP-TFII fragment into *Eco*RI/*Pst*I-digested pBluescript (Stratagene). The orientations and sequences of all mutants were confirmed by DNA sequencing. To generate COUP-TFII/The COUP-TFIIΔDBD, the N terminus coding sequence of COUP-TFII was amplified by PCR with a primer (CCGCTTCCCG TCTCACGCACTCGATGTG) corresponding to COUP-TFII cDNA sequences that preceded the DNA binding domain, whereas the C terminus coding sequence of COUP-TFII was amplified with a primer (CATCGAGTGCGTGAG ACGGGAAGCGGTG) corresponding to COUP-TFII cDNA sequences right after the DNA binding domain. The resulting PCR products were then ligated to generate a COUP-TFII mutant with the DNA binding domain deleted. The COUP-TFII cDNA deletion mutants were also cloned into $pCDNA_3$ (Invitrogen) for a transient transfection assay.

Preparation of COUP-TF proteins. Receptor proteins for COUP-TFI and COUP-TFII were synthesized by an in vitro transcription and translation system using rabbit reticulocyte lysate (Promega) as described previously (65). The relative amounts of the translated proteins were determined by using [^{35}S]methionine-labeled protein in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), quantitating the amount of incorporated radioactivity and normalizing it relative to the content of methionine in each protein.

Transient and stable transfection assay. CV-1 cells were plated at 10^5 cells per well in a 24-well plate 16 to 24 h before transfection, as described previously (66). For cancer cells, 5×10^5 cells were seeded in a six-well plate. A modified calcium phosphate precipitation procedure was used for transient transfection and is described elsewhere (66). Briefly, 700 ng of reporter plasmid, 100 ng of β-galactosidase (β-Gal) expression vector (pCH 110; Pharmacia), and various amounts of COUP-TF expression vector and RARα were mixed with carrier DNA (pBluescript) to 1,000 ng of total DNA per well. CAT activity was normalized for transfection efficiency to the corresponding β-Gal activity. For stable transfection, the pRc/CMV-COUP-TFI recombinant plasmid was stably transfected into MDA-MB231 cells, and pRC/CMV-antisense-COUP-TFI was stably transfected into J82 cells by the calcium phosphate precipitation method, and stable clones were screened with G418 (GIBCO BRL, Grand Island, N.Y.) as described previously (29). Southern blotting and Northern blotting were used to determine the integration and expression of transfected cDNA, respectively.

Gel retardation assay. Oligonucleotides used for the gel retardation assay are described in the text. For the protein-DNA binding assay, in vitro-translated protein (1 to 4 μl, depending on the translation efficiency) was incubated with the ^{32}P-labeled oligonucleotides in a 20-μl reaction mixture containing 10 mM HEPES buffer, pH 7.9, 50 mM KCl, 1 mM dithiothreitol, 2.5 mM $MgCl_2$, 10% glycerol, and 1 μg of poly(dI-dC) at 25°C for 20 min. Unprogrammed reticulocyte lysate was used to maintain equal protein concentrations in all reaction mixtures. Each reaction mixture was then loaded on a 5% nondenaturing polyacrylamide gel containing 0.5× TBE (1× TBE is 0.089 M Tris-borate, 0.088 M boric acid, and 0.002 M EDTA).

GST pull-down assay. To prepare the glutathione *S*-transferase (GST)-receptor fusion protein, the receptor cDNA (COUP-TFI or RARα) was cloned in frame into the expression vector pGEX-2T (Pharmacia). The fusion protein was expressed in bacteria by the procedure provided by the manufacturer and was analyzed by a gel retardation assay and Western blotting (data not shown). To analyze the interaction between RARα and COUP-TFI and between COUP-TFI and CBP, the fusion protein was immobilized to glutathione-Sepharose beads. For a control, the vector protein (GST), prepared under the same conditions, was also immobilized. The beads were preincubated with bovine serum albumin (1 mg/ml) at room temperature for 5 min. ^{35}S-labeled, in vitro-synthesized receptor proteins (2 to 5 μl, depending on translation efficiency) were then added to the beads. The beads were then continuously rocked for 1 h at 4°C in a final volume of 200 μl in EBC buffer (140 mM NaCl, 0.5% NP-40, 100 mM NaF, 200 μM sodium orthovanadate, and 50 mM Tris, pH 8.0). After being washed five times with NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.5% NP-40), the bound proteins were analyzed by SDS-PAGE.

Growth inhibition assays. To determine the effect of all-*trans*-RA on the viability of the stable transfectants, cells were seeded at 1,000 per well in a 96-well plate and treated with various concentrations of all-*trans*-RA for 6 days. Media were changed every 48 h. The number of viable cells was determined by MTT assay as described previously (29). For the anchorage-independent growth assay, 30,000 cells/60-mm-diameter dish in culture medium containing 10% FCS, 0.3% agar (Difco, Detroit, Mich.), and 10^{-7} M RA were plated onto an already-hardened 0.6% agar underlayer in medium supplemented with 10% FCS. The plates were incubated for 21 days in a 5% CO_2 incubator. Colonies with more than 40 cells were counted with a microscope.

Apoptosis assay. Cells were treated with or without all-*trans*-RA (10^{-6} M). Forty-eight hours later, cells were trypsinized, washed with phosphate-buffered saline (PBS; pH 7.4), and fixed in 1% formaldehyde in PBS. After being washed in PBS, cells were resuspended in 70% ice-cold ethyl alcohol and immediately stored at −20°C overnight. Cells were then labeled with biotin-16-dUTP by terminal deoxynucleotidyltransferase (TdT) and stained with avidin-fluorescein isothiocyanate (Boehringer, Mannheim, Germany). Fluorescently labeled cells were analyzed with a FACScater-plus as described previously (29). Representative histograms are shown.

Northern blotting. For Northern blot analysis, total RNAs were prepared with an RNeasy Mini Kit (Qiagen, Hilden, Germany). Thirty micrograms of total RNA from different cell lines treated with or without all-*trans*-RA (10^{-6} M) was analyzed by Northern blotting. An *Eco*RI fragment in the ligand binding domain of RARβ or a *Pst*I fragment in the ligand binding domain of COUP-TFI cDNA was used as a probe to study the expression of RARβ or COUP-TFII, respectively. The *Pst*I fragment could detect the expression of COUP-TFI and COUP-TFII due to the high degree of homology of both receptor genes in the region. To determine that equal amounts of RNA were used, the expression of β-actin was studied.

RESULTS

Correlation between COUP-TF expression and RARβ induction by RA. We recently showed that expression of COUP-TF is required for RA sensitivity in certain lung cancer cell lines (61). To examine to what degree COUP-TF is involved in the regulation of RARβ expression, we analyzed the expression of COUP-TF in various cancer cell lines, including breast cancer cell lines ZR-75-1, MDA-MB468, MDA-MB231, and T-47D, bladder cancer cell lines J82, HT-1376, 5637, and SCaBER, and lung cancer cell lines H520, H292, Calu-6, H460, H596, A-549, H441, SK-MES-1, and H661. A *Pst*I-*Bam*HI fragment in the ligand binding domain of COUP-TF was used as a probe. The probe could detect transcripts for COUP-TFI and COUP-TFII. When the expression of COUP-TFs was compared with RA-induced RARβ expression, we found a perfect correlation between COUP-TFI expression and the ability of RA to induce RARβ in breast cancer cell lines (Fig. 1). COUP-TFI was expressed in ZR-75-1 and T-47D cell lines, in which RARβ expression was highly induced by RA. In contrast, COUP-TF transcripts were not detected in MDA-MB468 and MDA-MB231 cell lines that did not show a clear induction of RARβ by RA. In bladder cancer cell lines, RARβ was highly induced by RA in COUP-TFI-positive J82 cells but not in COUP-TFI-negative 5637 and SCaBER cells. Although HT-1376 cells expressed COUP-TFI, we did not observe any RARβ expression. This is due to lack of RARα expression in these cells (see Discussion). A correlation was also observed in lung cancer cell lines, except SK-MES-1 and H661 lines.

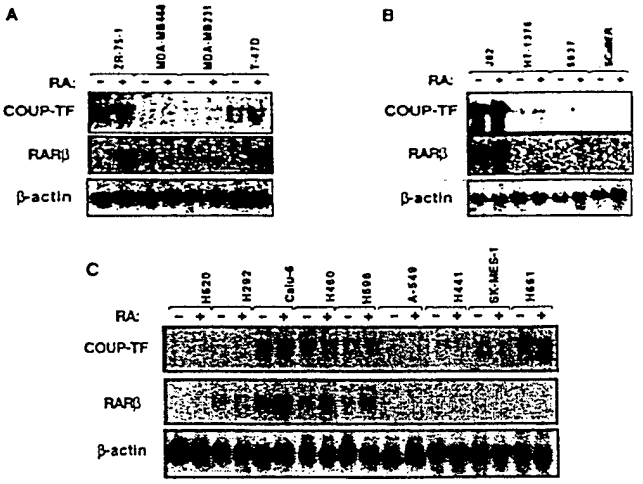

FIG. 1. Correlation between COUP-TF expression and RARβ induction by RA in human lung cancer, breast cancer, and bladder cancer lines. Total RNAs were prepared from the indicated cancer cell lines treated with or without all-*trans*-RA (10^{-6} M) for 24 h and analyzed for the expression of COUP-TFI and RARβ. For a control, the expression of β-actin is shown.

COUP-TFI and COUP-TFII were highly expressed in RARβ-inducible cell lines Calu-6, H460, and H596 but not in RARβ-noninducible H520, H292, A549, and H441 cell lines. These observations suggest that expression of COUP-TF may be required for RA to induce RARβ expression in different types of cancer cells.

Levels of COUP-TFI expression modulate RA-dependent RARβ expression, growth inhibition, and apoptosis. To further determine the requirement of COUP-TF for RA-dependent activation of RARβ gene expression, we stably expressed COUP-TFI in COUP-TF-negative MDA-MB231 breast cancer cells. Two stable clones (MB231/COUP#10 and MB231/COUP#16) that expressed a high level of transfected COUP-TFI (Fig. 2A) were subjected to an analysis of RARβ gene expression in the absence or presence of RA. Under the conditions used, we did not detect any expression of the RARβ transcript in the parental MDA-MB231 cells in either the absence or presence of RA (Fig. 2B), consistent with our previous observation (29). However, RA treatment strongly induced RARβ expression in the COUP-TFI-stable clones, not in the MDA-MB231 cells transfected with the empty vector (MB231/vector) (Fig. 2B). This suggests that expression of COUP-TFI confers on MDA-MB231 cells sensitivity to RA regulation of RARβ expression. When the effect of the stable transfection of COUP-TFI on growth of MDA-MB231 cells was analyzed, we observed that RA, which was unable to regulate the growth of the parental MDA-MB231 cells, could strongly inhibit the growth of the clones carrying stably transfected COUP-TFI, with about 41 and 54% inhibition in MB231/COUP#16 and MB231/COUP#10 cells, respectively, when they were treated with 10^{-6} M RA for 6 days (Fig. 2C). The growth-inhibitory effect of RA is specific to COUP-TFI expression since RA had no effect on the growth of MB231/vector cells. We also examined the effect of COUP-TFI expression on apoptosis induction by RA. The TdT assay showed extensive DNA fragmentation in MB231/COUP#10 and MB231/COUP#16 cells but not in MDA-MB231 and MB231/vector cells. About 2% apoptotic cells were detected in cultures of MDA-MB231 and MB231/vector cells when they were treated with 10^{-6} M RA for 2 days. However, the same RA treatment resulted in about

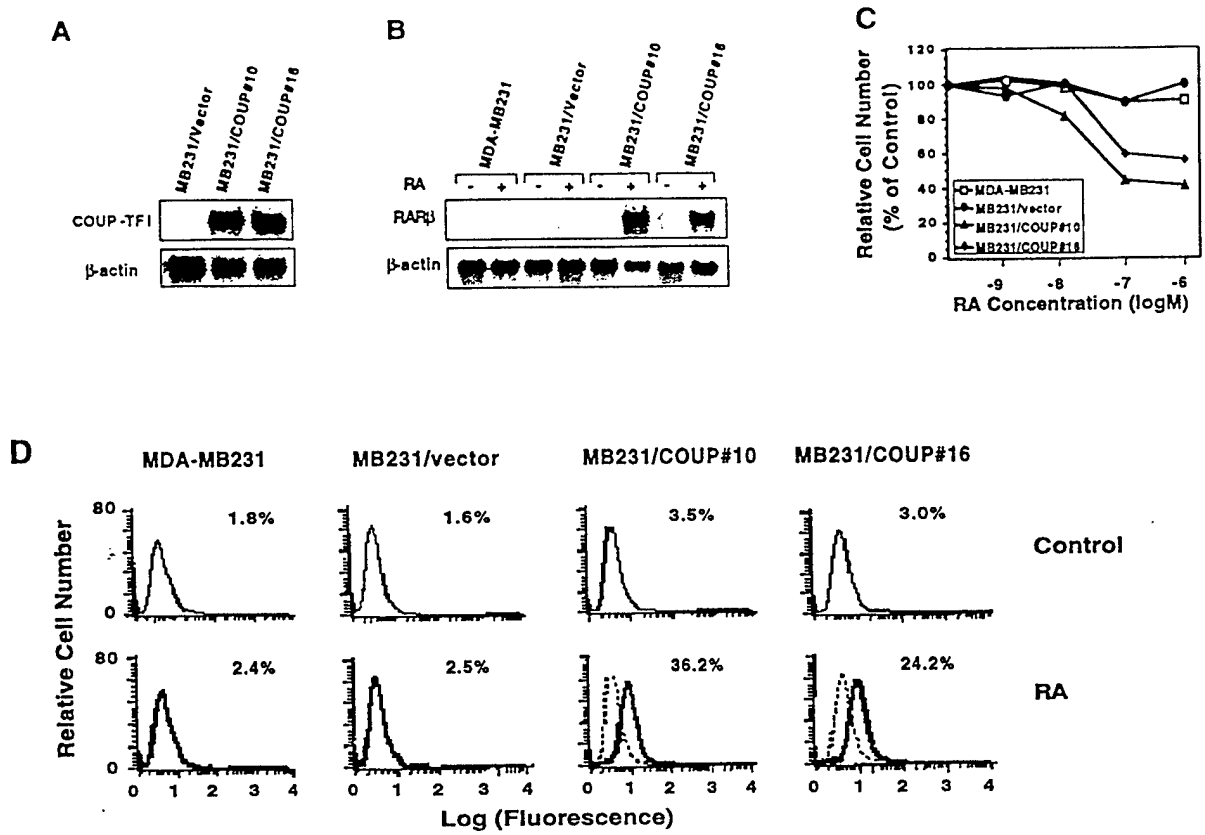

FIG. 2. Stable expression of COUP-TF in COUP-TF-negative, RA-resistant MDA-MB231 cells enhances the effect of RA on RARβ induction, growth inhibition, and apoptosis induction. (A) Expression of transfected COUP-TFI in MDA-MB231 cells. COUP-TFI was stably transfected into MDA-MB231 cells, and expression of transfected COUP-TFI was determined by Northern blotting. (B) Expression of RARβ gene in MDA-MB231 cells and MDA-MB231/COUP-TF stable clones. Total RNAs were prepared from MDA-MB231, its COUP-TFI stable clones (MB231/COUP#16 and MB231/COUP#10), and MDA-MB231 transfected with the empty vector (MB231/vector). Cells were treated with or without all-*trans*-RA (10^{-6} M) for 24 h and analyzed for the expression of RARβ by Northern blotting. In the control, the expression of β-actin is shown. (C) Growth-inhibitory effect of RA in MDA-MB231 and MDA-MB231/COUP-TF stable clones. Cells were seeded at 1,000 cells per well in a 96-well plate and treated with the indicated concentrations of all-*trans*-RA for 6 days. The numbers of viable cells were determined by the MTT assay. (D) Effect of RA on apoptosis of MDA-MB231 cells and MDA-MB231/COUP-TF stable clones. Cells were treated with or without all-*trans*-RA (10^{-6} M) for 48 h, and DNA fragmentations were then determined by the TdT assay. Representative histograms show relative apoptotic cell numbers.

36 and 24% apoptotic cells in cultures of MB231/COUP#10 and MB231/COUP#16 cells, respectively (Fig. 2D), suggesting that expression of COUP-TFI could allow RA to induce apoptosis of MDA-MB231 cells. Thus, expression of COUP-TFI in COUP-TF-negative cancer cells can restore their response to RA effects on RARβ expression, growth inhibition, and apoptosis.

To further study the role of COUP-TF, we stably expressed COUP-TFI antisense cDNA in COUP-TF-positive J82 bladder cancer cells. A stable clone that expressed a high level of COUP-TFI antisense RNA (J82/A-COUP) (Fig. 3A) was analyzed for the effect of RA on RARβ expression (Fig. 3B), growth inhibition (Fig. 3C), and apoptosis induction (Fig. 3D). RARβ expression was highly induced by RA in J82 cells. However, the ability of RA to induce RARβ expression was significantly reduced in J82/A-COUP cells but not in J82 cells expressing the empty vector (J82/vector) (Fig. 3B). Thus, inhibition of COUP-TF expression reduced the ability of RA to induce RARβ expression. When the effect of RA on growth inhibition was analyzed, we observed that RA could effectively inhibit the growth of J82 cells, with 55% inhibition when they were treated with 10^{-6} M RA for 6 days. A similar growth-inhibitory effect of RA (46% inhibition) was also observed in J82/vector cells. However, the effect of RA was dramatically reduced in J82/A-COUP cells, with only 12% inhibition upon RA treatment (Fig. 3C). Consistent with the role of RARβ in apoptosis induction, we observed that RA strongly induced apoptosis of J82 and J82/vector cells, with 43.8 and 36.2% apoptotic cells, respectively, as determined by the TdT assay (Fig. 3D). However, the apoptosis-inducing effect of RA was significantly repressed by COUP-TFI antisense RNA expression, as only 13.8% apoptotic cells were detected among J82/A-COUP cells. Together, these data demonstrate that inhibition of COUP-TFI expression by COUP-TFI antisense RNA represses the ability of RA to induce RARβ expression, growth inhibition, and apoptosis in COUP-TF-positive cancer cells.

To further characterize the effect of the transfected COUP-TFI gene, the COUP-TFI-transfected MDA-MB231 cells were analyzed for their anchorage-independent growth in soft agar. As shown in Fig. 4, the growth of MDA-MB231 and MB231/vector cells in soft agar was not clearly affected by RA treatment. However, RA dramatically inhibited the growth of MB231/COUP#10 and MB231/COUP#16 cells. These data suggest that expression of COUP-TFI could confer on RA the ability to inhibit the transforming activity of cancer cells.

COUP-TF enhances RARβ promoter activity in a RARα- and RA-dependent manner. To investigate the possibility that COUP-TFI induced RARβ expression by activating RARβ

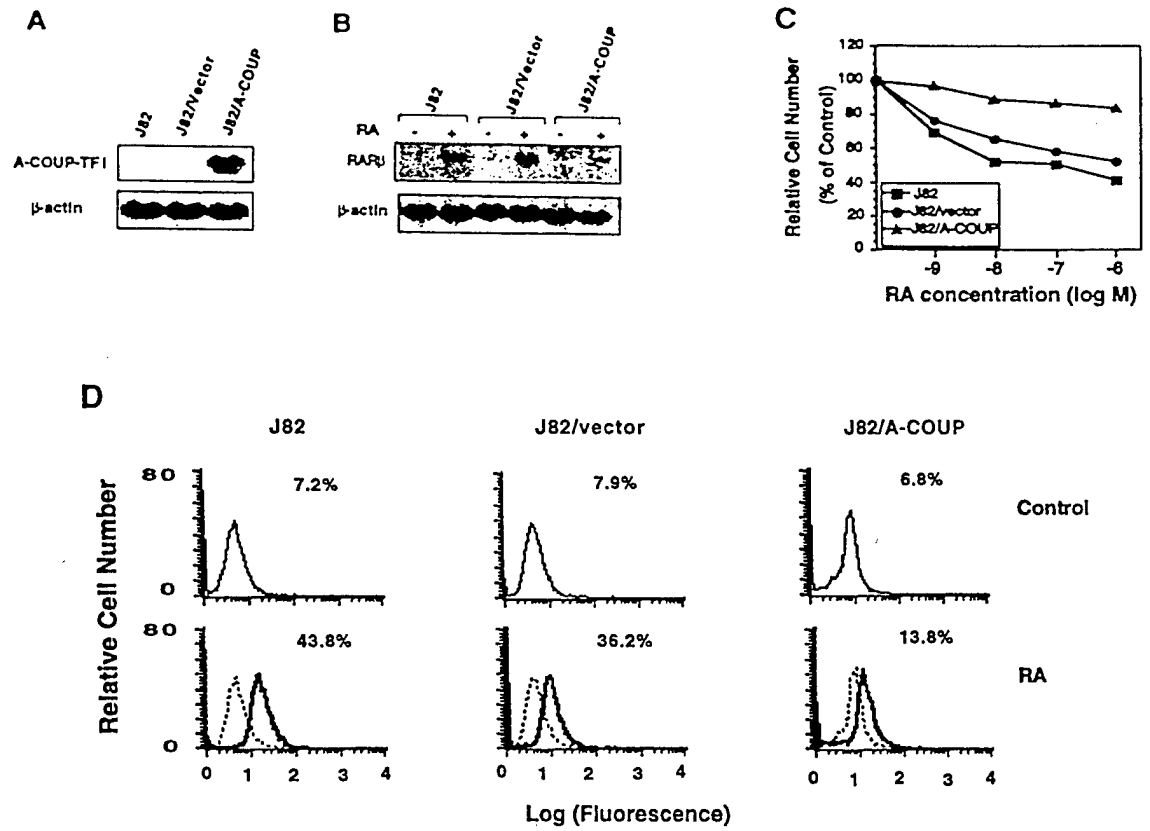

FIG. 3. Inhibition of COUP-TF expression by stable expression of COUP-TF anti-sense RNA in COUP-TF-positive J82 cells represses effect of RA on RARβ expression, growth inhibition, and apoptosis induction. (A) Expression of transfected COUP-TFI antisense RNA in J82 cells. COUP-TFI cDNA was stably transfected into J82 cells, and expression of transfected COUP-TFI antisense RNA in a selected stable clone (J82/A-COUP) was analyzed by Northern blotting. For comparison, the parental J82 cells and J82 cells stably transfected with the empty vector (J82/vector) were used. (B) Inhibition of RARβ induction by RA by stable expression of COUP-TFI antisense RNA in J82 cells. Total RNAs were prepared from J82, J82/Vector, and J82/A-COUP cells treated with or without all-*trans*-RA (10^{-6} M) for 24 h and analyzed for the expression of RARβ by Northern blotting. The expression of β-actin is shown for the control. (C) Inhibition of RA-induced growth inhibition by stable expression of COUP-TF antisense RNA in J82 cells. Cells were seeded at 1,000 cells per well in a 96-well plate and treated with the indicated concentrations of all-*trans*-RA for 6 days. The numbers of viable cells were determined by the MTT assay. (D) Inhibition of RA-induced apoptosis of J82 cells by COUP-TF antisense RNA. Cells were treated with or without all-*trans*-RA (10^{-6} M) for 48 h, and DNA fragmentations were then determined by the TdT assay. Representative histograms show relative apoptotic cell numbers.

promoter transcription, a reporter construct that contains the RARβ promoter sequences from −745 to +162 linked to the CAT reporter gene (−745RARβCAT) (13) was transiently transfected into CV-1 cells. Cotransfection of the RARα expression vector induced RARβ promoter activity in response to RA (Fig. 5A), while cotransfection of the COUP-TFII expression vector slightly enhanced the reporter gene activity. However, when both COUP-TFII and RARα were cotransfected, a synergistic induction of RARβ promoter activity in response to RA was observed. Induction of RARβ promoter activity by RARα was enhanced from 16-fold to 44-fold when 20 ng of COUP-TFII was cotransfected, while a 56-fold induction was observed when 50 ng of COUP-TFII was cotransfected. A similar observation was also made with COUP-TFI (data not shown). We also investigated the effect of COUP-TFII in HT-1376 bladder cancer cells, which express a low level of COUP-TF (Fig. 1) and an undetectable level of RARα (our unpublished result). Cotransfection of the RARα expression vector slightly induced RARβ promoter activity in response to RA in these cells, while cotransfection of COUP-TFII did not show any effect on RARβ promoter activity (Fig. 5B). The lack of COUP-TFII activity is likely due to loss of RARα expression in these cells (see Discussion). When COUP-TFII was cotransfected with RARα, the RARα-induced RARβ activity was strongly increased, compared to a 5-fold induction of RARβ promoter activity by RARα alone and a 24-fold induction when both RARα and COUP-TFII were present. Similar results were also obtained when COUP-TFI was used and in other cancer cell lines, such as HeLa cells and MDA-MB231 cells (data not shown). Together, our data demonstrate that expression of COUP-TF is required for efficient induction of RARβ promoter activity by RA in a RARα-dependent manner.

A DR-8 element in the RARβ promoter mediates the COUP-TF effect. To identify DNA sequences responsible for the COUP-TF effect in the RARβ promoter, we generated a series of 5′ deletion mutants of the RARβ promoter (Fig. 6A). The resulting RARβ promoter fragments were fused to the CAT reporter gene and analyzed for the effect of COUP-TFII in enhancing RA-induced RARα activity in CV-1 cells (Fig. 6B). Deletion of 229 bp from the 5′ end of the RARβ promoter (−516RARβCAT) did not have any effect on the ability of COUP-TFII to enhance RARβ activity. Recently, it was shown that COUP-TF could positively regulate gene transcription through an SP-1 site (42, 46). There is an SP-1 site (from −378 to −370) in the RARβ promoter that could bind the

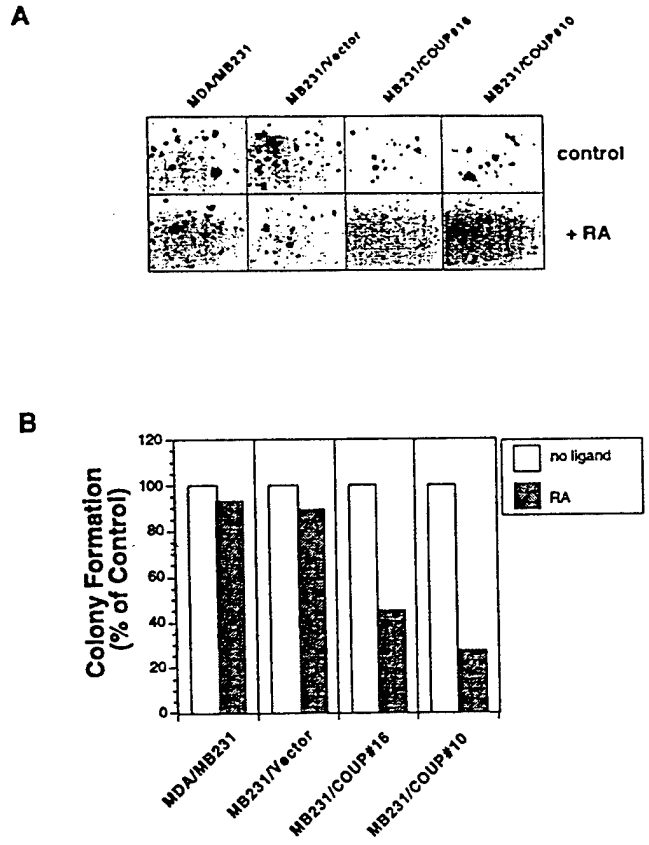

FIG. 4. Inhibition of anchorage-independent growth of MDA-MB231 cells by COUP-TF gene expression. (A) Visualization of colonies formed in the soft agar by parental MDA-MB231, MB231/COUP#10, MB231/COUP#16, and MB231/vector cells in the presence or absence of RA (10^{-7} M). (B) Quantitation of colonies formed by parental MDA-MB231, MB231/COUP#10, MB231/COUP#16, and MB231/vector cells. Colonies formed by MB231/COUP#10, MB231/COUP#16, MB231/vector, and parental MDA-MB231 cells in the presence or absence of all-*trans*-RA (10^{-7} M) were scored and expressed as percentages of colonies formed by cells treated with control solvent.

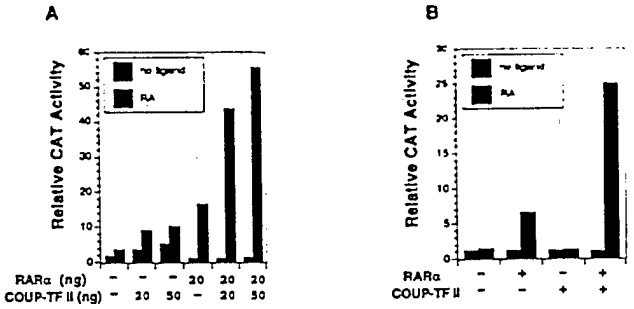

FIG. 5. COUP-TF enhances RARβ promoter activity in a RARα- and RA-dependent manner. (A) Activation of RARβ promoter activity by COUP-TF in CV-1 cells. RARβ promoter reporter (−745RARβCAT; 700 ng) was cotransfected with the indicated amounts of expression vector for COUP-TFII and RARα into CV-1 cells. Cells were treated with or without all-*trans*-RA (10^{-6} M) for 24 h and assayed for CAT activity. (B) Activation of RARβ promoter in HT-1376 bladder cancer cells by COUP-TF. Cells were transfected with 1,500 ng of −745RARβCAT reporter gene together with expression vectors for RARα (300 ng) and/or COUP-TFII (300 ng). Cells were treated with or without all-*trans*-RA (10^{-6} M) and 24 h later were assayed for CAT activity. Data shown represent the means of three independent experiments.

SP-1 protein (data not shown). However, COUP-TFII retained its ability to induce RARα activity in −300RARβCAT and −126RARβCAT reporters in spite of deletion of the SP-1 binding site. This suggests that the effect of COUP-TFII on the RARβ promoter is not mediated through the SP-1 binding site. Further deletion of 66 bp from −126RARβCAT, however, completely abolished the COUP-TFII effect (Fig. 6B). This suggests that position −126 to −60 of the RARβ promoter contains a COUP-TF response element (COUP-TF-RE). Inspection of the fragment between positions −126 and −60 revealed a direct repeat of AGGTCA-like motifs with 8-bp spacing (DR-8) located from −99 to −78 (Fig. 7A). COUP-TF is known to bind to a variety of nuclear receptor response elements containing AGGTCA-like motifs arranged in different orientations with different spacings (53). We therefore examined whether the DR-8 element in the RARβ promoter could bind to COUP-TF. An oligonucleotide containing the sequence was synthesized and analyzed by gel retardation assay for its COUP-TF binding activity. As shown in Fig. 7B, in vitro-synthesized COUP-TFI or COUP-TFII formed a strong complex with the sequence. For comparison, RARα, RXRα, or the RARα-RXRα heterodimer did not exhibit any binding. The complex formed by COUP-TFI could be abolished by a 50-fold-excess amount of TREpal or CRBPII-RARE, which are known to bind with high affinity to COUP-TFs (53), while the same amount of SP-1 oligonucleotide did not show any effect on the binding. Thus, COUP-TF may exert its effect on the RARβ promoter through its binding to the DR-8 element.

To study the role of the COUP-TF binding sequence, we investigated the effect of COUP-TF-RE mutations on COUP-TF activity in a RARβ promoter. The COUP-TF-RE was mutated by changing either the spacing between two core motifs (COUP-TF-RE/M1) or the core motif sequences (COUP-TF-RE/M2) (Fig. 8A). The mutated COUP-TF-REs were first analyzed for their binding to COUP-TF proteins. Unlike their binding to the wild-type COUP-TF-RE, the binding of COUP-TFI or COUP-TFII to the mutated COUP-TF-REs was largely impaired (Fig. 8B). We then introduced the same mutations into the −745RARβCAT reporter by PCR. The resulting RARβ promoter reporter mutants, −745RARβ/COUP-TF-RE/M1/CAT and −745RARβ/COUP-TF-RE/M2/CAT, were then analyzed by transient transfection assay for the effect of COUP-TF-RE mutations on COUP-TFII activity. As shown in Fig. 8C, mutations of COUP-TF-RE did not affect the ability of RARα to induce reporter transcription since cotransfection of RARα showed similar degrees of induction of transcription of both the mutated RARβ promoter and the wild-type promoter (compare with Fig. 5). However, the enhancing effect of COUP-TFII on RARα activity was largely reduced in the mutated RARβ promoter reporters. These results demonstrate that the DR-8 element is required for COUP-TF to enhance RA- and RARα-dependent activation of RARβ promoter transcription.

DNA binding of COUP-TF is required for its transactivation function. To determine which region of COUP-TF is required for the binding of its DNA to the DR-8 element and the activation of the RARβ promoter, a number of COUP-TFII mutants were generated (Fig. 9A) and analyzed for their binding to the element (Fig. 9B) and their enhancing effect on RARα activity in the −745RARβCAT reporter (Fig. 9C). Deletion of seven amino acids from the C-terminal end of the COUP-TFII protein did not affect the binding of its DNA to COUP-TF-RE (Fig. 9B). The same mutant also bound approximately as strongly to TREpal as to the wild-type receptor. However, removal of an additional seven amino acid residues

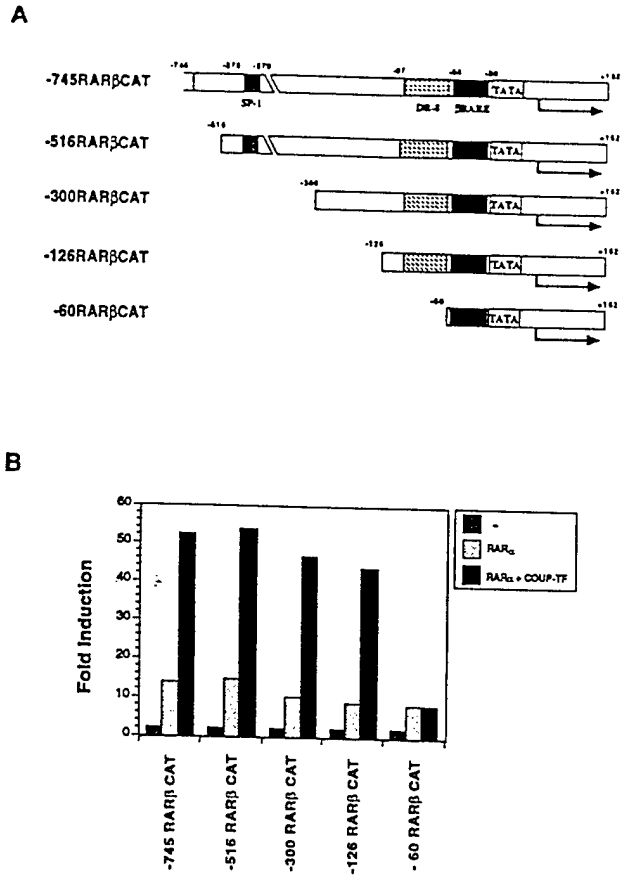

FIG. 6. Identification of COUP-TF response element in the RARβ promoter. (A) Schematic representation of the RARβ promoter deletion mutants. The Sp-1 binding site, βRARE, and the TATA box are indicated. (B) Effect of COUP-TFII on RA-induced RARα transactivation activity of various RARβ promoter deletion mutants. CV-1 cells were transfected with 700 ng of CAT reporter gene containing the indicated RARβ promoter fragment together with expression vectors for COUP-TFII (50 ng) and RARα (20 ng). Cells were then treated with or without all-*trans*-RA (10^{-6} M) and 24 h later were assayed for CAT activity. Data shown represent the means of three independent experiments.

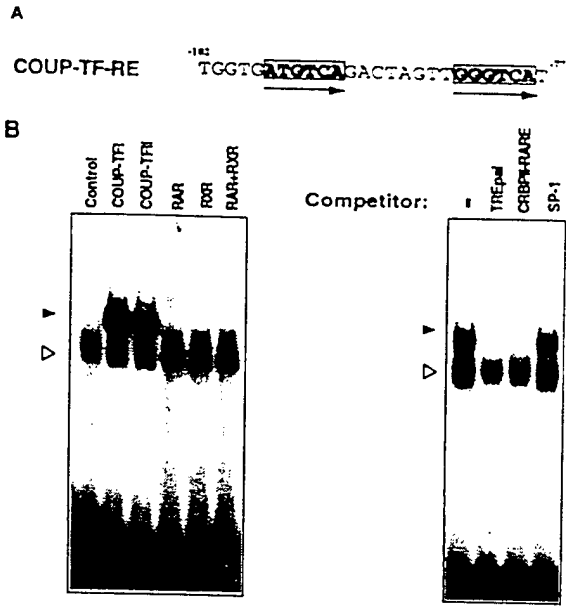

FIG. 7. Analysis of COUP-TF binding on the COUP-TF-RE in the RARβ promoter. (A) Sequence of the COUP-TF-RE. Arrows indicate the AGGTCA-like core motifs. (B) Binding of COUP-TF on the COUP-TF-RE. In vitro-synthesized COUP-TFI or COUP-TFII was incubated with ^{32}P-labeled oligonucleotide containing the COUP-TF-RE and analyzed by a gel retardation assay. For comparison, the binding of RARα, RXRα, and the RARα-RXRα heterodimer was analyzed. For the competition assay, a 50-fold excess amount of the indicated oligonucleotide was used.

resulted in a mutant (COUP-TFII/Δ14) that could not bind either to COUP-TF-RE or to TREpal. As expected, other mutants analyzed, including COUP-TFII/Δ30, COUP-TFII/Δ80, COUP-TFII/Δ108, and COUP-TFII/Δ179 (Fig. 9A), did not exhibit any detectable binding on both elements (Fig. 9B). When the effect of these mutants on RARα transactivation function in −745RARβCAT was analyzed, we observed that removal of as few as 14 amino acid residues from the C-terminal end of the COUP-TFII protein completely abolished the enhancing effect of COUP-TFII (Fig. 9C). Other C-terminal deletion mutants, which could not bind to the DR-8 element, also failed to enhance RARα activity (data not shown). A mutant with the DNA binding domain deleted (COUP-TF/ΔDBD) was also unable to enhance RARα activity (Fig. 9C). Thus, the DNA binding of COUP-TF is essential for its enhancing effect on RARα activity.

βRARE is required for the DR-8 element to confer the RA- and RARα-dependent transactivation function of COUP-TF. To further determine the role of the DR-8 element in mediating the COUP-TF transactivation function, we cloned the sequence into the pBLCAT$_2$ plasmid, which contains the thymidine kinase (TK) promoter linked with the CAT reporter gene (65). The resulting construct, COUP-TF-RE-tk-CAT, was transiently transfected into CV-1 cells. Surprisingly, we did not see any effect of COUP-TFII on reporter transcription either in the absence or presence of RA, indicating that the binding of COUP-TFII could not transactivate the reporter (Fig. 10). Cotransfection of the RARα expression vector did not have any effect on the reporter activity, either in the absence or presence of RA, consistent with our gel retardation assay showing lack of RARα binding to the DR-8 element (Fig. 7B). Furthermore, cotransfection of COUP-TFII and RARα expression vectors failed to activate reporter transcription. These data demonstrate that the DR-8 element alone is not sufficient to confer the RA- and RARα-dependent transactivation function of COUP-TF observed in the RARβ promoter.

The above data suggest that sequences other than the DR-8 element in the RARβ promoter are required for the RA- and RARα-dependent transactivation function of COUP-TFs. We therefore analyzed the involvement of βRARE by mutational analysis since it is the only known sequence in the RARβ promoter that mediates the RA effect (13). Two βRARE mutations were made by changing either the core motif sequences (βRARE/M1) or the spacing between two core motifs (βRARE/M2) (Fig. 11A). Both βRARE mutants failed to bind to RARα, RXRα, or RARα-RXRα heterodimers in the gel retardation assay (Fig. 11B). The RARβ promoter reporter constructs with mutated βRARE, −745RARβCAT/βRARE/M1 and −745RARβCAT/βRARE/M2, were then generated by PCR and analyzed by a transient transfection assay of CV-1 cells (Fig. 11C). We first examined the effect of RARα on the mutated RARβ promoter. Compared to that of the parental RARβ promoter (−745RARβCAT), the transactivation function of RARα was significantly reduced in the

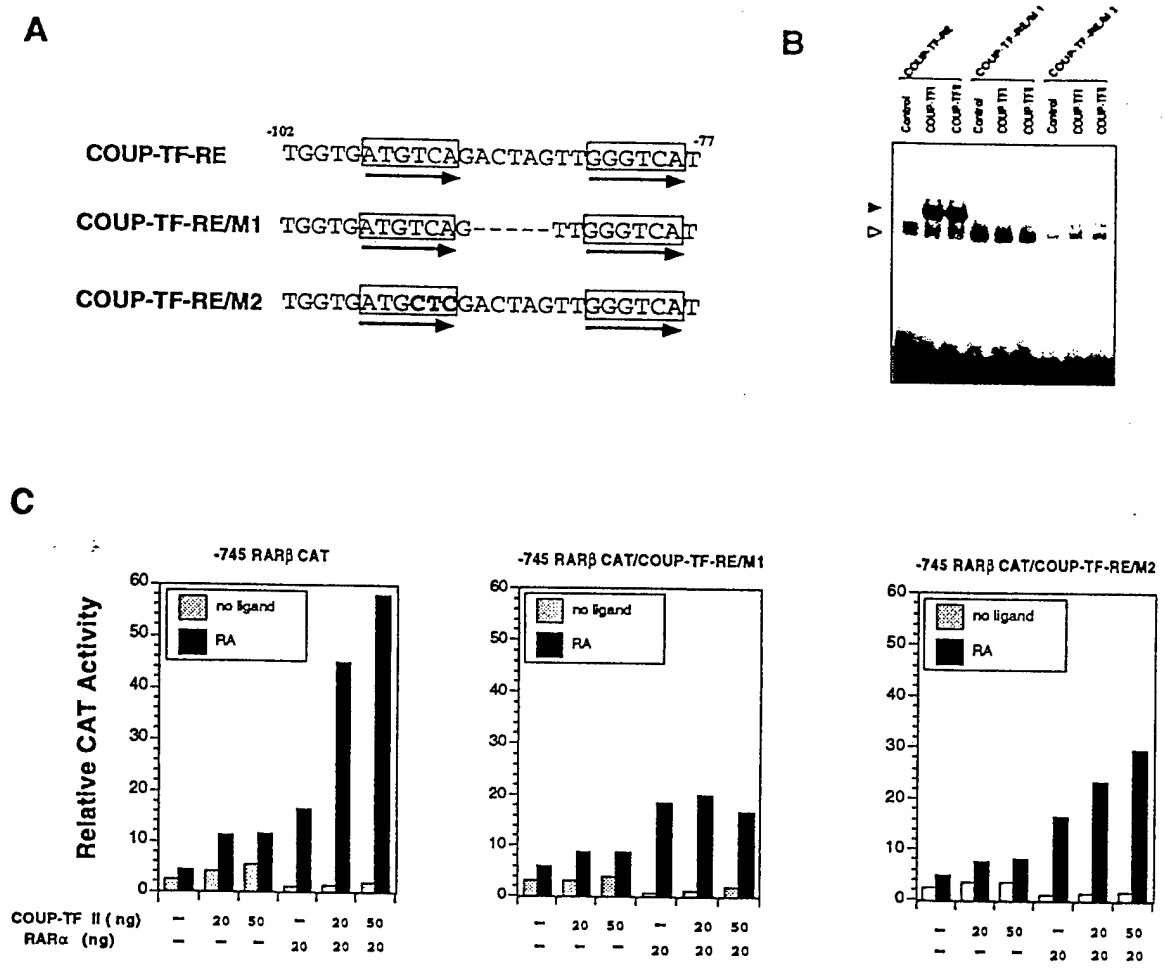

FIG. 8. COUP-TF-RE is required for positive regulation of RARβ promoter activity by COUP-TF. (A) Mutations of the COUP-TF-RE. Depicted are the COUP-TF-RE sequence and its mutations. (B) The mutated COUP-TF-REs failed to bind to the COUP-TF protein. The indicated COUP-TF-RE mutant was synthesized and analyzed for its binding to COUP-TF by the gel retardation assay. In vitro-synthesized COUP-TF protein was incubated with ^{32}P-labeled COUP-TF-RE or the mutated COUP-TF-RE and analyzed by a gel retardation assay. (C) Mutations of COUP-TF-RE abolished the effect of COUP-TF on the RARα transactivation function in the RARβ promoter. The COUP-TF-RE in the RARβ promoter was mutated by PCR. The resulting RARβ promoter mutants (−745RARβCAT/COUP-TF-RE/M1 and −745RARβCAT/COUP-TF-RE/M2) were analyzed by transient transfection assay in CV-1 cells for their effect on COUP-TF activity. CV-1 cells were transfected with the 700-ng reporter gene together with the indicated amounts of expression vectors for RARα and COUP-TFII. Cells were treated with or without all-*trans*-RA (10^{-6} M) and 24 h later were assayed for CAT activity. CAT activity was normalized for transfection efficiency to the corresponding β-Gal activity. Data shown represent the means of three independent experiments.

mutated RARβ promoters (−745RARβCAT/βRARE/M1 and −745RARβCAT/βRARE/M2). Interestingly, RARα could still induce transcriptional activities of both RARβ promoter mutants, although its binding on the mutated βRAREs was completely abolished (Fig. 11B). Since we did not observe any effect of RARα on the mutated βRAREs when they were fused to the TK promoter (data not shown), the observed effect of RARα is likely due to the presence of another RARE in the RARβ promoter (our unpublished observation). Nevertheless, when the effect of COUP-TFII on both promoter mutants was analyzed, we did not observe any enhancement of RARα activity, suggesting that intact βRARE, capable of binding with the RARα-RXRα heterodimer, is essential for the transactivation function of COUP-TF. Thus, both βRARE and the DR-8 element are required for the RA- and RARα-dependent transactivational function of COUP-TF in the RARβ promoter.

COUP-TF enhances recruitment of CBP by RARα. The transactivation function of nuclear receptors requires their interaction with receptor coactivators (62), such as CBP (18). The transactivation function of COUP-TF could be due to its interaction with CBP. We therefore investigated whether COUP-TFI could interact with CBP by the GST pull-down assay. Under the conditions used, we did not observe a clear interaction between COUP-TFI and CBP (Fig. 12A), consistent with a previous report (44). This demonstrates that the transactivation function of COUP-TFI is unlikely to be mediated through its direct interaction with CBP. The facts that the transactivation function of COUP-TF is RARα and RA dependent and requires βRARE suggest that the effect of COUP-TF may be mediated by RARα, which is known to interact with CBP (18). We then incubated GST–COUP-TFI with CBP in the presence of in vitro-synthesized RARα. As shown in Fig. 12A, a significant amount of CBP was pulled

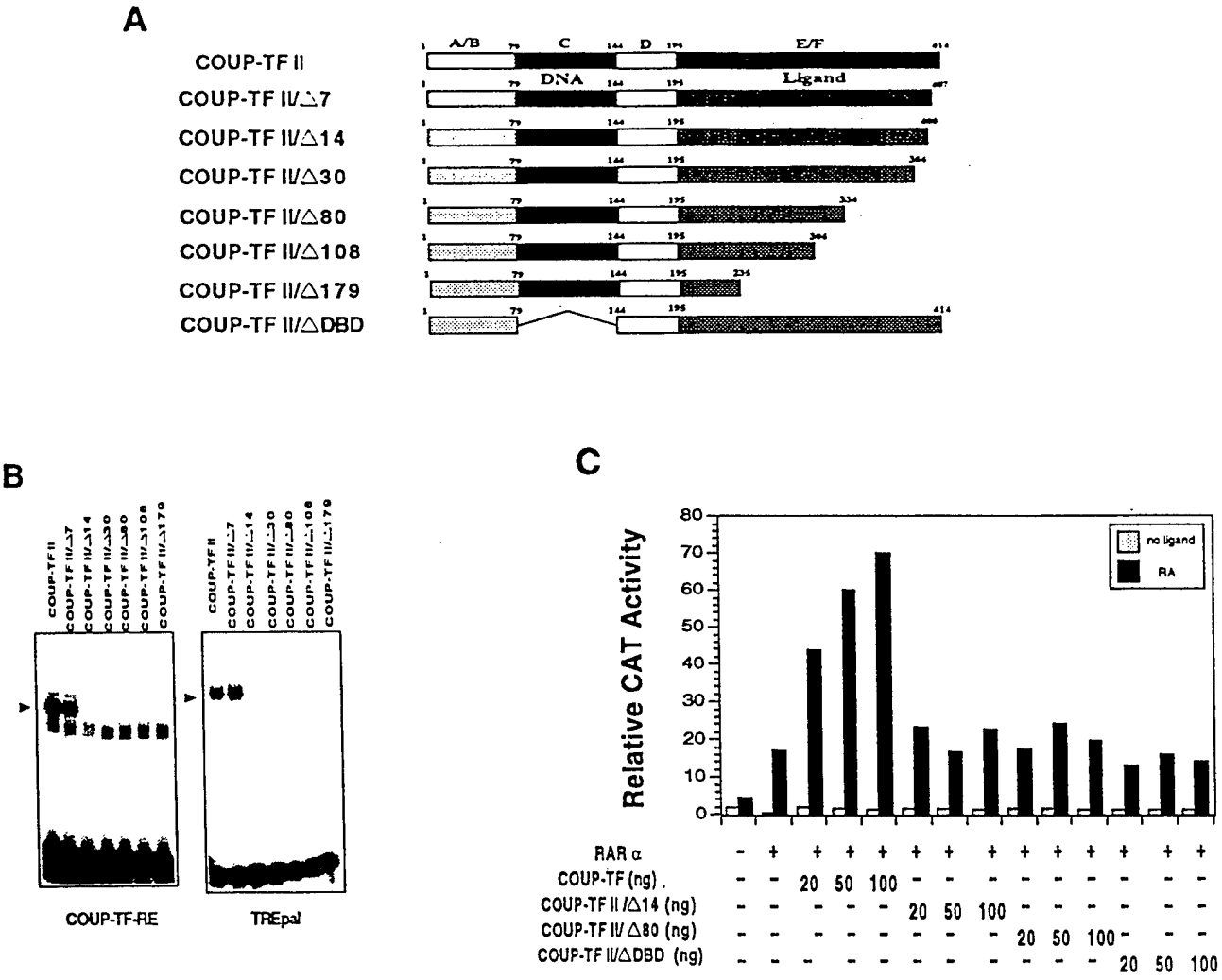

FIG. 9. DNA binding of COUP-TF is required for its enhancing effect on RARα activity in the RARβ promoter. (A) Schematic representation of COUP-TF mutants. The DNA binding domain and the ligand domain of COUP-TFII are indicated. (B) Binding of the COUP-TFII mutants to COUP-TF-RE and TREpal. In vitro-synthesized COUP-TFII or the indicated COUP-TFII deletion mutant was incubated with ^{32}P-labeled COUP-TF-RE or TREpal as indicated and analyzed by a gel retardation assay. The arrowheads indicate the specific COUP-TF binding complexes. (C) Effect of the COUP-TFII mutants on RARα activity in the RARβ promoter. CV-1 cells were transfected with 700 ng of −745RARβCAT reporter gene together with the expression receptor for RARα (20 ng) and the indicated amount of COUP-TFII or COUP-TFII deletion mutants. Cells were treated with or without all-*trans*-RA (10^{-6} M) for 24 h and were assayed for CAT activity. Data shown represent the means of three independent experiments.

down by the GST–COUP-TFI fusion protein when RARα was present. We also determined whether COUP-TF could enhance the interaction between RARα and CBP. As shown in Fig. 12A, CBP was slightly pulled down by the GST-RARα fusion protein. However, when GST-RARα was mixed with in vitro-synthesized COUP-TFI protein, a significant amount of CBP was pulled down. The observation that both RARα and COUP-TFI are required for their maximum interaction with CBP suggests that COUP-TFI may interact with RARα. Indeed, in vitro-synthesized RARα was efficiently pulled down by GST–COUP-TFI, but not by the GST control protein (Fig. 12B), demonstrating that COUP-TFI could directly interact with RARα. Thus, COUP-TF may facilitate the RARα-CBP interaction by interacting with RARα, resulting in a RARα conformation favorable for CBP interaction.

We next analyzed whether COUP-TF could influence the transcriptional effect of CBP on RARα activity in CV-1 cells. Cotransfection of the CBP expression vector slightly enhanced RARα transcriptional activity in the RARβ promoter. However, when COUP-TFII and RARα were cotransfected, the effect of CBP on RARβ promoter activity was greatly increased (Fig. 12C). Such an effect of COUP-TF on CBP activity, together with our GST pull-down results, strongly suggests that COUP-TF, through its binding to the DR-8 element and interacting with RARα, may function as a bridge protein to facilitate the interaction between βRARE binding receptors and CBP, resulting in an enhanced RA-dependent transcription of the RARβ promoter.

DISCUSSION

RARβ plays a crucial role in the mediating growth-inhibitory effect of retinoids, and lack of its expression contributes to retinoid resistance in many different types of cancer cells. Expression of RARβ is induced by RA through binding of RAR-RXR heterodimers to the βRARE in the RARβ promoter.

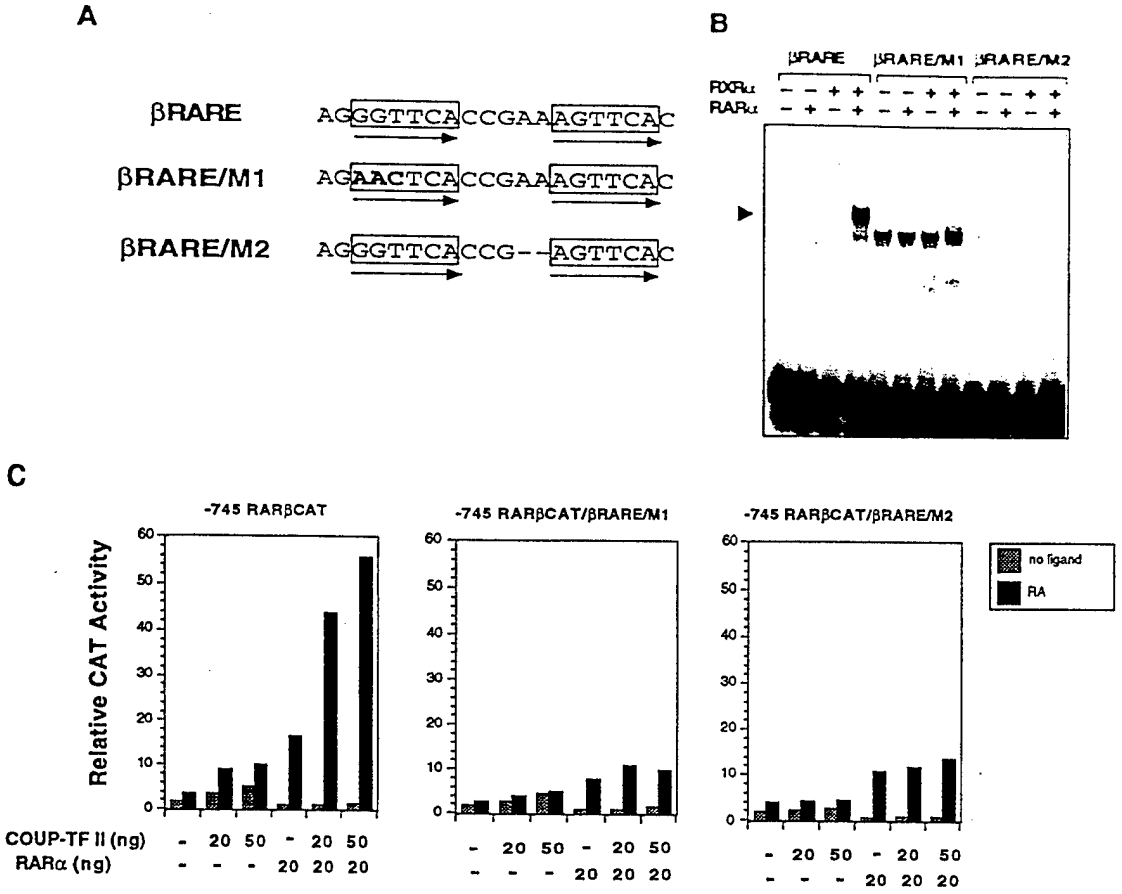

FIG. 11. βRARE in the RARβ promoter is required for the enhancing effect of COUP-TF on RARα activity in the RARβ promoter. (A) Schematic representation of the βRARE mutations. Depicted are wild-type βRARE in the RARβ promoter and its mutations. (B) Effect of βRARE mutations on retinoid receptor binding. In vitro-synthesized RARα and RXRα were incubated with ^{32}P-labeled βRARE or the mutated βRARE and analyzed by a gel retardation assay. The arrowhead indicates specific RAR-RXR heterodimer binding. (C) Mutations of the βRARE impair the enhancing effect of COUP-TF on RARα activity in the RARβ promoter. Mutations in the βRARE of the RARβ promoter were introduced by PCR as described in Materials and Methods. The resulting RARβ promoter mutants (−745RARβCAT/βRARE/M1 and −745RARβCAT/βRARE/M2) were analyzed by transient transfection assay for the effect of COUP-TF. CV-1 cells were transfected with 700 ng of reporter gene together with the indicated amounts of expression vectors for COUP-TFII and RARα. Cells were then treated with or without all-*trans*-RA (10^{-6} M) and 24 h later were assayed for CAT activity. Data shown represent the means of three independent experiments.

that βRARE is required for the positive transactivation function of COUP-TF is interesting in light of the previous observation that COUP-TF is an effective negative regulator of RAREs (3, 20, 53, 59). Recently, Folkers et al. reported (7) that COUP-TF could inhibit the activity of βRARE and the RARβ promoter due to its binding to the element. However, the notion that COUP-TF acts as a negative regulator of the βRARE was based mainly on transient transfection assays where COUP-TF might be overexpressed. In fact, induction of βRARE and RARβ promoter activity was observed when a low concentration of COUP-TF was used (7). This is consistent with our previous study (61) showing that COUP-TF, at low concentrations, enhanced the RA sensitivity of the βRARE through its binding to the element, which prevents RA-independent activation of the βRARE. These observations demonstrate that COUP-TF at appropriate concentrations, which are likely to occur in most cells, does not act as a negative regulator of the βRARE and the RARβ promoter. This is well supported by observations that RARβ and COUP-TF are coexpressed in various cancer cell lines (Fig. 1) and motor neurons (33, 48). Thus our present data, together with our previous findings (61), demonstrate that COUP-TF plays a critical role in the regulation of RA-dependent RARβ expression by acting as an activator and sensitizer through its binding to COUP-TF-RE and βRARE, respectively.

COUP-TF acts as an accessory protein for RARα. Results provided in this study demonstrate a new mechanism for positive gene regulation by COUP-TF. Unlike the effect of COUP-TF on vHNF1 (45), human immunodeficiency virus long terminal repeat (49), and NGFI-A (44), which does not require COUP-TF DNA binding, DNA binding of COUP-TF is essential for COUP-TF to positively regulate RARβ gene expression (Fig. 8 and 9). The positive transcriptional regulation observed in the NGFI-A gene is mediated through a direct transactivation function of COUP-TF through its recruitment of receptor coactivator SRC-1 (44). However, the effect of COUP-TF on the RARβ promoter is not mediated by a direct transactivation function of COUP-TF since cotransfection of COUP-TF could not activate transcription of the COUP-TF-RE-tk-CAT reporter (Fig. 10). Our observation that cotransfection of COUP-TF alone could not activate the RARβ promoter in HT-1376 cells (Fig. 5) due to the lack of RARα expression in the cells (data not shown) demonstrates that the transactivation function of COUP-TF in the RARβ promoter is likely mediated by RARα. This is further supported by our finding that positive gene regulation by COUP-TF was RARα

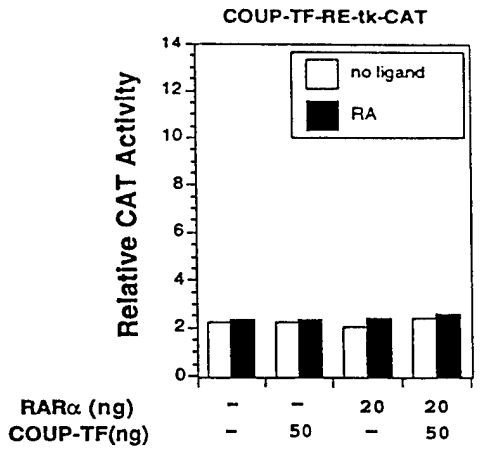

FIG. 10. COUP-TF-RE alone is not sufficient to confer the activation function of COUP-TF. COUP-TF-RE was cloned into pBLCAT$_2$, which contained the TK promoter linked with the CAT gene. The resulting reporter construct (COUP-TF-RE-tk-CAT) was analyzed for its response to the COUP-TF effect by transient transfection assay. The reporter gene (100 ng) was cotransfected with the indicated amounts of expression receptor for RARα and COUP-TFII into CV-1 cells. After transfection, cells were treated with or without all-*trans*-RA (10^{-6} M) and 24 h later were assayed for CAT activity. Data shown represent the means of three independent experiments.

However, expression of RAR and RXR in cancer cells is not sufficient to account for induction of RARβ by RA. Here, we provide convincing evidence that orphan receptor COUP-TF is required for RA to induce RARβ expression, growth inhibition, and apoptosis in cancer cells and that loss of COUP-TF expression is the main factor contributing to the lack of RARβ expression in cancer cells. In addition, we show that COUP-TF synergistically increases the RA-dependent RARα transactivation function in the RARβ promoter through its binding to a DR-8 element in the promoter, which enhances the interaction of RARα with its coactivator CBP.

Expression of COUP-TF is required for efficient RARβ induction by RA in cancer cells. Several lines of evidence provided in this study demonstrate that expression of COUP-TF is required for efficient RARβ induction by RA in cancer cells. First, expression of COUP-TF is positively correlated with induction of RARβ by RA in breast cancer, lung cancer, and bladder cancer cell lines (Fig. 1). A perfect correlation was observed in breast cancer cell lines, while a close correlation was found in lung cancer and bladder cancer cell lines. This observation is consistent with previous studies (33, 47, 48) demonstrating that RARβ and COUP-TFII are coexpressed in motor neurons. In addition, our stable expression of COUP-TFI in COUP-TF-negative MDA-MB-231 breast cancer cells (Fig. 2) showed that expression of COUP-TFI in these cell lines could restore the ability of RA to induce RARβ expression. Furthermore, inhibition of COUP-TF expression by stable expression of COUP-TFI antisense RNA in COUP-TF-positive J82 cancer cells reduced the ability of RA to induce RARβ expression (Fig. 3). Finally, our transient transfection assays clearly demonstrated that COUP-TF could activate the RARβ promoter in response to RA when RARα was expressed (Fig. 5). Thus expression of COUP-TF is required for RA to effectively induce RARβ expression, and loss of COUP-TF in cancer cells may be one of the important mechanisms responsible for the lack of RARβ expression in cancer cells.

Effect of COUP-TF on growth inhibition and apoptosis induction by RA. RA is known to inhibit the growth of and induce apoptosis in cancer cells, in part due to its induction of RARβ (27–29). Our observation that COUP-TF expression is required for RARβ induction by RA implies that COUP-TF is also involved in RA-induced growth inhibition and apoptosis signaling. This is clearly shown by our stable transfection of COUP-TFI in COUP-TF-negative MDA-MB231 cells (Fig. 2). MDA-MB231 breast cancer cells are RA resistant due to lack of RARβ induction by RA (28). When COUP-TFI was stably expressed in these cells, their growth was strongly inhibited by RA (Fig. 2C). Furthermore, the cells underwent extensive apoptosis in response to RA treatment (Fig. 2D). The observation that RARβ was strongly induced in the COUP-TFI stable clones (Fig. 2B) suggests that the observed growth inhibition and apoptosis induction are likely mediated by the induced RARβ expression. The involvement of COUP-TF in the regulation of RA-dependent growth inhibition and apoptosis induction is further supported by our stable expression of COUP-TFI antisense RNA in COUP-TF-positive J82 bladder cancer cells (Fig. 3). Expression of COUP-TFI antisense RNA in this RA-sensitive cell line dramatically reduced the ability of RA to induce growth inhibition (Fig. 3C) and apoptosis of the cells (Fig. 3D), which was accompanied by a reduced level of RARβ expression (Fig. 3B). Together, our results demonstrate that COUP-TF may play a critical role in the regulation of the growth-inhibitory effect of retinoids in cancer cells.

Transactivation function of COUP-TF requires both COUP-TF-RE and βRARE in the RARβ promoter. By mutation analysis, we demonstrated that a DNA sequence comprising two AGGTCA-like core motifs arranged as a direct repeat with 8-bp spacing (the DR-8 element) is required for the COUP-TF effect. The DR-8 element bound strongly with COUP-TF (Fig. 7). Interestingly, the binding of COUP-TF did not repress the RARβ promoter. Instead, it is essential for the transactivation function of COUP-TF. Deletion of the sequence from the RARβ promoter completely abolished the effect of COUP-TF on RARβ promoter activity (Fig. 6). In addition, mutations in the DR-8 element that impaired the binding of COUP-TF reduced the COUP-TF effect on the enhancement of RARα activity (Fig. 8). The requirement of COUP-TF DNA binding is also supported by our COUP-TF mutational analysis showing that deletion of as few as 14 amino acid residues from the C-terminal end of COUP-TF abolished both its DNA binding and transactivation function (Fig. 9). Furthermore, COUP-TF with the DNA binding domain deleted was unable to enhance RARβ promoter activity (Fig. 9). Thus, unlike its binding to RAREs, which results in repression of RARE activity (13, 20, 53, 59), the binding of COUP-TF to the DR-8 element in the RARβ promoter is required for COUP-TF to enhance RA-dependent RARα activity. Interestingly, the binding of COUP-TF to a DR-7 element in the arrestin gene promoter could mediate the positive transcriptional effect of COUP-TF (32). Thus, whether COUP-TF exerts transactivation or transrepression function is largely dependent on the configuration of its DNA binding sequences.

RARE alone is sufficient to confer the repressive effect of COUP-TF on the element (20, 53, 60). However, our results demonstrate that the DR-8 element in the RARβ promoter alone is not sufficient to confer the transactivation function of COUP-TF. When it was fused to the TK promoter, the DR-8 element could not be activated by COUP-TF in the presence or absence of RARα (Fig. 10). Thus, the effect of COUP-TF is specific to the RARβ promoter. By mutational analysis (Fig. 11), we demonstrated that the βRARE in the RARβ promoter is required for DR-8 to mediate COUP-TF. The observation

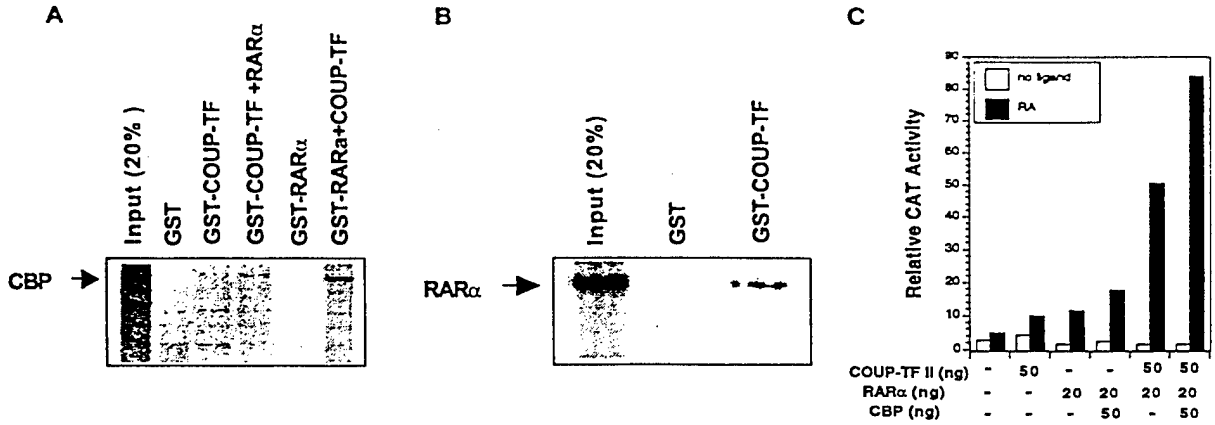

FIG. 12. COUP-TF enhances recruitment of CBP by RARα. (A) COUP-TF enhances interaction of RAR and CBP. COUP-TFI or RARα protein was synthesized in bacteria with pGEX-2T (Pharmacia). The GST–COUP-TFI or GST-RARα fusion protein was immobilized on glutathione-Sepharose beads, while the same amount of GST was also immobilized on beads as a control. ^{35}S-labeled CBP was then mixed with beads and in vitro-synthesized RARα (for GST–COUP-TFI) or in vitro-synthesized COUP-TFI (for GST-RARα) in the presence of 10^{-6} M all-*trans*-RA. After extensive washing, the bound proteins were analyzed by SDS-PAGE. The input proteins are shown for comparison. (B) COUP-TFI interacts with RARα. To analyze the interaction between COUP-TF and RARα, ^{35}S-labeled RARα was mixed with GST–COUP-TFI fusion protein on beads. After extensive washing, the bound proteins were analyzed by SDS-PAGE. As a comparison, the input proteins are shown. (C) COUP-TF increases the effect of CBP on RARα activity. The indicated amounts of expression vector for RARα, COUP-TFII, and CBP were cotransfected with RARβ promoter into CV-1 cells. Cells were treated with or without all-*trans*-RA (10^{-6} M) for 24 h and then were assayed for CAT activity. The corresponding β-Gal activity was normalized as a control.

dependent (Fig. 5). Indeed, COUP-TF did not show a clear interaction with the receptor coactivator CBP (Fig. 12), consistent with a previous report (44). However, COUP-TF strongly enhances the interaction between RARα and CBP (Fig. 12A). This may explain the enhancement of the transcriptional effect of CBP by COUP-TF on RA-dependent RARα activity (Fig. 12C). It is likely that COUP-TF, through its direct interaction with RARα (Fig. 12B), may induce a RARα conformation change that is more favorable for its interaction with CBP. Thus, COUP-TF functions as an accessory protein for RARα to activate the RARβ promoter. It may also be considered a member of the RARα coactivator complex on the RARβ promoter.

In summary, studies described here reveal a novel mechanism by which COUP-TF positively regulates RA-induced RARβ expression through its ability to interact with RARα and the DR-8 element in the RARβ promoter. The observation that the expression of COUP-TF is crucial for RARβ expression and anticancer activities of RA further enhances our understanding of the retinoid signaling in cancer cells. This becomes especially apparent since retinoid resistance is frequently observed in various types of cancer cells despite expression of functional retinoid receptors (19, 52, 65). Our finding that COUP-TF is not expressed in a majority of RA-resistant cancer cell lines (Fig. 1) suggests that the loss of COUP-TF may represent one of the important mechanisms for retinoid resistance in cancer cells. It also indicates that an understanding of how COUP-TF expression is lost may be crucial for understanding defects associated with retinoid resistance in cancer cells. The fact that loss of RARβ expression is an early event in breast carcinogenesis (41, 57, 60) implies that COUP-TF may also play a role in cancer development.

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